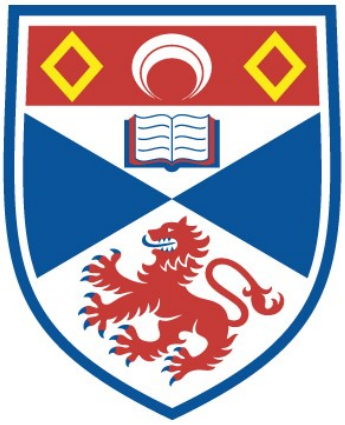
THE PREPARATION, PROPERTIES AND APPLICATIONS OF NYLON-TUBE IMMOBILISED CATALASE

Stuart D. Ashworth

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE PREPARATION, PROPERTIES AND APPLICATIONS OF NYLON-TUBE IMMOBILISED CATALASE

by

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A thesis submitted to the University of St. Andrews in application for the degree of Doctor of Philosophy.

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Declaration

I hereby declare that the following thesis is based on my own work, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry of the University of St. Andrews, under the direction of Dr. W.E.Hornby.

Certificate

I hereby declare that Stuart D. Ashworth has spent nine terms in research work under my direction and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that he is qualified to submit this thesis for the degree Doctor of Philosophy.

Academic Record

I matriculated at the University of St. Andrews in October 1968, and graduated with the degree Bachelor of Science, First Class Honours in Biochemistry in June 1972.

In October 1972, I matriculated as a research student at the University of St. Andrews.

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I should like to thank my supervisor, Dr. W.E.Hornby for his advice and criticism throughout this work.

Thanks are also due to Mr E.Sanders for his endless patience throughout the past three years.

Contents

Abstract

1. Several nylon-tube immobilised catalase derivatives were prepared. The method of preparation of nylon-tube for enzyme attachment was varied in an attempt to obtain the most active immobilised catalase derivative.

2. The stability of nylon-tube immobilised catalase derivatives during constant perfusion with hydrogen peroxide was examined in an attempt to prepare the most stable immobilised enzyme.

3. The peroxidatic activity associated with nylon-tube immobilised catalase derivatives was investigated. The effect of the chemistry of immobilisation, the length and diameter of the nylon-tube, and methanol concentration on the peroxidatic : catalatic activity ratio displayed by the immobilised catalase derivatives.

4. The applications of nylon-tube immobilised catalase derivatives in automated analysis was considered. Glucose was determined by four different assay protocols, involving 1) nylon-tube co-immobilised glucose oxidase and catalase ; 2) nylon-tube immobilised derivatives of glucose oxidase and catalase in series ; 3) nylon-tube immobilised derivatives of glucose oxidase, catalase, and aldehyde dehydrogenase in series ; 4) nylon-tube co-immobilised glucose oxidase and catalase in conjunction with an oxygen electrode.

5. The peroxidatic activity of catalase was monitored using

a) Hantzsch reaction ; b) Aldehyde dehydrogenase ; and

c) flow-through oxygen electrode.

6. The four analytical systems were calibrated with aqueous glucose standards and the assay sensitivity and immobilised enzyme stability determined.

7. Comparisons were drawn between the four methods for glucose determination using nylon-tube immobilised enzymes.

ABBREVIATIONS

A	=	Diethyladipimidate
AH	=	Adipic acid dihydrazide
alk	=	Alkylated
ATP	=	Adenosine triphosphate
Az	=	Azide-activated
DAE	=	1,2-Diaminoethane
DMS	=	Dimethyl sulphate
E	=	Ethanolamine
G	=	Glutaraldehyde
HMDA	=	1,6-Diaminohexane
Lys	=	L-Lysine
NAD	=	Nicotinamide adenine dinucleotide
1Nt	=	1 mm bore nylon-tube alkylated using triethyloxonium tetrafluoroborate
2Nt	=	2 mm bore nylon-tube alkylated using triethyloxonium tetrafluoroborate
SH	=	Succinic acid dihydrazide
TOTFB	=	Triethyloxonium tetrafluoroborate
Tris	=	Tris(hydroxymethyl)methylamine

CHAPTER 1

INTRODUCTION

A satisfactory way to define immobilised enzymes is in terms of their operational characteristics. Thus immobilised enzymes can be considered as enzymes that have been modified in such a way that the enzyme protein is in a phase separate to that of both the substrates and products and hence can easily be recovered from the reaction mixture.

Enzyme immobilisation can be performed by a variety of methods including adsorption of the enzyme onto an insoluble material ; occlusion within a gel-matrix ; microencapsulation within a semi-permeable microcapsule ; or covalent attachment to an insoluble support. Thus in the past twenty years over sixty enzymes have been immobilised onto one or more of forty different support structures using the above methods(1).

Arguably the simplest method for preparing immobilised enzymes involves the adsorption of the enzyme onto the surface of a suitable support. This can be achieved by incubating the enzyme with the support, whereupon either hydrophobic or ionic bonding will occur, depending upon the nature of the support. However as this process is of a physical nature the possibility of enzyme detachment exists. For example, an immobilised enzyme of this type may be susceptible to protein losses due to such variation in reaction conditions as pH, ionic strength and temperature.

In many ways gel inclusion of the enzyme is a more satisfactory procedure for the preparation of immobilised enzymes. In this method the enzyme is incubated with a gel-monomer (acrylamide is often used) and a matrix is formed by cross-linking of the monomers. By variation of the extent of cross-linking, it is possible to provide a matrix in which the pore size is too small to allow the enzyme to escape, but large enough for the substrate to gain access to the enzyme and products

to escape from the matrix. However in some cases the mechanical stability of the gel matrix is limited. As any deterioration of the matrix results in enzyme leakage, care has to be exercised in the handling of such immobilised enzymes. Leakage of enzyme can also be caused by the breakdown of matrix over a prolonged period of use (70).

Possibly the best method for immobilising enzymes is to attach the enzyme to a suitable support through covalent bonds. This procedure can be effected in several ways (1,3,4). In general, the enzyme can be immobilised via an active group present on the support. This can be achieved by either the activation of a previously inert support (4) or the synthesis of a support incorporating the necessary groups required for enzyme attachment (71). In either case, the enzyme can be attached directly to the support or through cross-linking reagents such as glutaraldehyde or bis-imidates. Enzymes immobilised in this manner are generally irreversibly bound to the support and therefore little trouble is experienced with detachment of the enzyme. However, protein losses have been reported from enzymes immobilised in this way, and this has been explained by losses in structural integrity of the support material rather than rupture of covalent bonds (12).

The specificity and speed of enzymic conversions has ensured a great potential for these catalysts in both industrial and analytical applications. Already enzymes are being used on a fairly large scale in both fields at the present time. For example, proteases are used in the manufacture of some dairy and food products ; glucose oxidase and catalase in the removal of glucose from food products ; and lipases in the rendering of some fats. For several reasons, however, the full potential of enzymes has not been realised as yet. Costs prevent the

use of many enzymes in large scale preparative procedures as the soluble enzyme can only be used once, recovery procedures being far too complicated and costly to be practicable. This inability to recover soluble enzyme also means that the product is contaminated with extraneous protein - in all likelyhood from a non-mammalian source - and therefore problems can arise due to standard health and safety regulations.

The use of immobilised enzymes successfully overcome these problems as recovery of the enzyme is possible by simple and quick means such as filtration or centrifugation. Insoluble proteases are used in the manufacture of foods and beverages, and have been reported as satisfactory milk-curdling agents (5). Immobilised L-amino acid acylases are in use in the large-scale resolution of D- and L- amino acids while immobilised penicillin amidases have been used in the production of 6-amino penicillinic acid (5).

In a field of analysis enzymes are also becoming far more widely used. Their substrate specificity makes them ideal candidates for the monitoring of compounds such as glucose, urea, uric acid, cholesterol and many other biologically significant compounds occurring in serum or urine. The use of more conventional methods however is still widespread owing to their low operating cost. These procedures are generally based upon intrinsic chemical properties associated with the substance under assay. For example, the majority of glucose determinations performed in analytical laboratories utilise the reducing properties of glucose. In alkaline conditions glucose will reduce cupric ions to cuprous ions with the formation of cuprous oxide. This can be detected by its reaction with neocuproine (2,9-dimethyl-1,10-phenanthroline hydrochloride). In this procedure, as with many other non-enzymic analyses results usually give slightly

higher values than when compared with enzymic assays (9). In many cases this is due to the presence of interfering substances in the sample which affect the chemical reaction. Enzymic assays, while not always completely specific tend to produce more reliable results.

The enzymic determination of glucose levels in biological fluids has been proposed (7,8). These methods use soluble enzymes, for example, in standard Technicon autoanalytical systems. While relatively crude and cheap preparations of these enzymes are employed, improvements could be made by increasing the cost-effectiveness and simplicity of the systems. The cost of several other enzymes important in clinical analysis (for example urate oxidase and cholesterol oxidase) prohibit their use in a soluble form in continuous flow analysis. Before these systems can be used for comprehensive screening of large quantities of samples the immobilisation of these enzymes is a neccessity.

The potential of some immobilised enzymes in clinical analysis has been investigated previously. For example, Guilbault has used immobilised enzymes in the form of enzyme electrodes for the determination of blood constituents such as glucose and urea (72,73). Hicks and Updike have employed immobilised glucose oxidase in the form of a packed-bed reactor for the determination of glucose in the so-called "Reagentless" assay (74). Hornby et al. have immobilised enzymes on the inside surface of nylon and polystyrene tubes in order to determine such metabolites as glucose, urea and ethanol (4,39-41). The use of immobilised enzymes in clinical analysis has recently been reviewed (75).

For automated analytical procedures in continuous flow systems, it is imperative to be able to incorporate immobilised enzyme into an automated system capable of performing a large number of assays per hour.

For this reason, many of the standard supports employed for immobilisation are completely inapplicable for this type of usage. The need to prevent cross-contamination of samples in a flow-line, possibly containing up to fifteen samples under assay, requires air-segmentation of the liquid throughout the system. Supports such as membranes, gels, and packed-bed reactors are not suitable for use in existing systems. The tubular-shaped support is ideally suited for such a system as its structural compatibility allows the free passage of air-segmented reagents, and maintains discrete samples. Enzymes have been immobilised previously upon glass and nylon-tubes (4,6). The feasability of using nylon-tube and polystyrene-tube immobilised enzymes for routine clinical analysis has been reported previously (10,11).

Therefore when considering possible applications of immobilised enzyme derivatives, the choice of the type of support and its morphology are of great importance. As previously mentioned, enzymes have been immobilised to over forty different support structures. The properties of enzymes immobilised to thesedifferent supports often vary with the structure of the support material. For example, it has been suggested that enzymes immobilised to naturally occurring polymers such as Sepharose and cellulose tend to display a decreased storage life and increased sensitivity to thermal denaturation (12), whereas enzymes immobilised to synthetic polymers such as nylon and glass, are more resistant to bacterial contamination but may display reduced activity. In general, reports indicate that immobilised enzyme display more stability than the corresponding enzyme in a soluble form (3,13).

The physical nature of the support material also is of importance. Tubular supports, although suited for clinical analysis are probably of limited use in industry as the percentage conversion of substrates

per pass would be too small. Immobilisation of enzymes onto powders with their employment in either packed-bed reactors or slurries produces far greater enzymic activity owing to the vastly increased surface area of the support, and consequently these supports are favoured in industrial processes. For example, Warburton et al. have used immobilised penicillin amidase in a continuous feed stirred tank reactor to convert benzylpenicillin to 6-amino penicillinic acid (14).

The chemistry of attachment of the enzyme can also affect the suitability of the immobilised derivative to perform a specific function. For example, the introduction of charge onto the support material can affect non-specific adsorption of substrates or products onto the support (15). This is especially important when considering the role of immobilised enzymes in clinical analysis. Such adsorption can severely limit the rate at which samples can be analysed owing to cross-contamination of the samples. It can also affect the pH optimum of the derivative by the formation of an artificial micro-environment due to the partitioning of hydrogen ions by the support (9).

Thus careful consideration of all the properties of the support material is necessary in the formulation of an immobilised enzyme derivative for a specific purpose.

By virtue of its catalatic and peroxidatic activities catalase is one enzyme that has great potential in an immobilised form in both industrial and diagnostic fields. Its conversion of hydrogen peroxide to oxygen and water without the neccessity of the addition of a further substrate, makes it of great importance in the removal of hydrogen peroxide from food-stuffs after sterilisation. The use of the soluble enzyme for this purpose is not always suitable due to the resulting contamination of the material with protein. This problem

can be obviated by the use of an immobilised catalase derivative. However, previous derivatives using such supports as cheese-cloth, glass, kieslguhr and silica alumina particles have been found to be rapidly deactivated by hydrogen peroxide (16-20). Therefore the lack of stability of these derivatives has been a disadvantage when considering their applications in the removal of hydrogen peroxide.

Catalase has also been used in clinical analytical systems. The estimation of serum glucose levels has been performed using glucose oxidase and catalase immobilised upon both controlled pore glass beads and polyacrlamide matrices (21,22). In these systems, catalase acts catalatically thus preventing analytical errors arising from the presence of endogenous catalase in the sample. Catalase has also been used in a more fundamental role in clinical analysis. The peroxidatic "side-reaction" of the enzyme is capable of converting methanol, ethanol, and propanol (but not butanol nor any other ascending member of the homologous series) into the corresponding aldehydes (23). The determination of uric acid in serum and urine has been performed using soluble urate oxidase and catalase (24). The hydrogen peroxide formed by the urate oxidase is used in the peroxidatic conversion of methanol to formaldehyde by catalase. This latter reagent is monitored by means of the Hantzsch reaction (25). In theory this assay protocol, involving the peroxidatic activity of catalase, can be applied to any oxidase that is capable of producing hydrogen peroxide.

The soluble enzyme has been found to react with hydrogen peroxide in a variety of ways. The catalytically active complex of catalase and hydrogen peroxide (commonly termed Compound 1) can react with either another hydrogen peroxide molecule to form oxygen and water or with an

alcohol to form the corresponding aldehyde and water. This latter reaction is an example of the peroxidatic activity of catalase. However, it is also known that in the presence of hydrogen peroxide, and in the absence of any alternative hydrogen donors, catalase can also form complexes which are catalatically inactive. These are termed Compounds 2 and 3. Whereas Compound 3 is thought to be completely inactive, compounds such as phenols and sodium nitrite are capable of reducing Compound 2 to free catalase, by engaging in a single electron reaction (54). The reactions of catalase with hydrogen peroxide and alcohols are shown in Fig A.

$$\text{Cat (OH)}_4 + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Cat (OH)}_3\text{--OOH} + H_2O$$

$$\text{Cat (OH)}_3\,\text{OOH} + H_2O_2 \xrightarrow[k_4]{} \text{Cat (OH)}_4 + H_2O + O_2$$

$$\text{Cat (OH)}_3\,\text{OOH} + CH_3OH \xrightarrow[k_4]{} \text{Cat (OH)}_4 + HCHO + H_2O$$

$$\text{Cat (OH)}_3\,\text{OOH} \xrightarrow{k_7} \text{Compound II} \longrightarrow \text{Compound III}$$

Fig A. Reactions of Catalase.

The purpose of this present work is directed towards the applications of immobilised catalase in autoanalysis. As previously mentioned, a tubular-shaped support is ideally suited for such a purpose. Nylon was chosen as the support material owing to its great mechanical strength (which imparts a robustness on the immobilised enzyme) and its ready availability. The potential application of nylon-tube immobilised enzymes in clinical analysis have been reported previously (46). Nylon, being an inert polymer requires the generation of active groups prior to enzyme attachment. This has previously been

performed using acid hydrolysis, in order to liberate free amino groups (41). While this method enables enzymes to be bound to the nylon, the structural integrity of the support is partially lost owing to the hydrolytic cleavage of the amide linkage. However, activation of the nylon can be achieved without disruption of the secondary amide backbone of the polymer. Morris et al. (46) have reported the immobilisation of enzymes upon nylon-tube activated by reagents such as DMS and TOTFB. These reagents react with the secondary amide linkages of nylon to produce the corresponding secondary imidate salts. Enzymes may be attached directly to nylon prepared in this manner by the reaction of amine functions on the protein with the imidate groups generated upon the nylon. Alternatively, a spacer compound may be attached to the nylon-tube by means of the imidate groups. The enzymes can then be covalently bound to substituted nylon-tubes via cross-linking reagents such as glutaraldehyde and bis-imidates. This method of nylon activation not only ensures the maintenance of the structural integrity of the support but also allows a greater flexibility in the chemistry involved for enzyme immobilisation. Variation of the spacer compounds employed, enables the microenvironment of the immobilised enzyme to be changed, thus allowing the determination of optimisation or the environment most suited to the individual enzyme.

In order to ascertain the nylon-tube immobilised catalase derivative most suited for use in autoanalytical systems, the chemistry of attachment of the enzyme to the nylon-tube has been varied. In this way, the derivative displaying 1) the greatest stability to hydrogen peroxide, 2) the highest ratio of peroxidatic : catalatic activity and 3) the best operational performance in auto-analytical systems has been determined.

The determination of glucose has been investigated using four systems involving nylon-tube immobilised glucose oxidase and catalase derivatives. In two, the peroxidatic activity of the catalase-tube has been monitored by means of the Hantzsch reaction. The third system monitors the peroxidatic activity of the catalase tube by means of a third enzyme, aldehyde dehydrogenase. The last system monitors the glucose oxidase activity by means of oxygen depletion. In this latter case a flow-through oxygen electrode has been employed.

2.1. BUFFERS

Buffers of constant ionic strength were prepared from the data of Datta and Grzybowski (26). The pH of all buffers was checked using a Pye Dynacap pH meter (W.G. Pye & Co. Ltd., Cambridge, U.K.). Prior to use, this instrument was calibrated using a standard reference buffer of pH 6.86 at 25°C (Beckman Instruments Inc., Fullerton, California, U.S.A.).

2.2. SOLVENTS

Diethyl ether, methanol, dichloromethane, dioxan and formamide were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Before use all solvents were redistilled and stored over calcium hydride.

2.3. INORGANIC AND ORGANIC REAGENTS

Wherever possible, Analytical grade reagents were used without further purification. Except where otherwise stated such reagents were obtained from BDH Chemicals Ltd.

Borontrifluoride diethyletherate was stored in a dark bottle at room temperature, saturated with sulphur dioxide.

1-Chloro-2:3-epoxypropane was stored in a dark bottle at room temperature.

Glutaraldehyde, 25% (w/v), electron microscopy grade, was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and stored at 4°C.

Egg Albumen (crystallised and lyophilised, Sigma Chemical Co., (London) Ltd., Kingston upon Thames, Surrey, U.K.) was stored dessicated at 4°C. Solutions of this protein in the appropriate buffer were made up immediately prior to their use.

Solutions of L-lysine monohydrochloride and methylated albumen

were made up immediately prior to use. Solutions of 1,2-diaminoethane and 1,6-diaminohexane in methanol were stored at 4°C.

Formaldehyde was obtained from Koch-Light Laboratories Ltd., and stored at 4°C. Immediately prior to use, solutions of this reagent were standardised by their oxidation and back-titration with a primary standard of potassium iodide.

Acetylacetone was stored in the dark at room temperature.

Adiponitrile and adipamide were obtained from Cambrian Chemicals Ltd., Croydon, Surrey, U.K.

Type 6 nylon-tube was obtained from Portex Ltd., Hythe, Kent, U.K., and used throughout. Where thick bore nylon-tube is specified the internal diameter of the tube measured 2 mm. Thin bore nylon-tube had an internal diameter of 1 mm.

2.4. ENZYMES

All enzymes, were used without further purification. With the exception of glucose-6-phosphate dehydrogenase they were stored dessicated over silica gel at 4°C.

2.4.1 Catalase (EC 1.11.1.6)

Type C-40 catalase from beef-liver (15,000 Sigma Units mg^{-1}) was obtained from Sigma Chemical Co., Ltd.

B-grade catalase (9,500 IU mg^{-1}) from Aspergillus niger was obtained from Calbiochem Ltd., San Diego, California, U.S.A.

2.4.2 Glucose Oxidase (EC 1.1.3.4)

Grade 1 glucose oxidase (210 IU mg^{-1}) from Aspergillus niger was obtained from Boehringer Corporation London Ltd., Uxbridge, U.K.

2.4.3 Peroxidase (EC 1.11.1.7)

Type VI peroxidase (250 Purpurogallol U mg^{-1}) from horseradish was obtained from Sigma Chemical Co., Ltd.

2.4.4 Hexokinase (EC 2.7.1.1)

Type F300 hexokinase (210 IU mg^{-1}) from yeast was obtained from Boehringer Corporation, London, Ltd.

2.4.5 Glucose-6-phosphate Dehydrogenase (EC 1.1.1.49)

Grade II glucose-6-phosphate dehydrogenase (140 IU mg^{-1}) from yeast was obtained as a crystalline suspension from Boehringer Corporation, London, Ltd. and stored at $4^{o}C$.

2.4.6 Aldehyde Dehydrogenase (EC 1.2.1.5)

Grade II aldehyde dehydrogenase (6 IU mg^{-1}) from yeast was obtained from Sigma Chemical Co., Ltd.

2.4.7 Alcohol Dehydrogenase (EC 1.1.1.1)

Alcohol dehydrogenase (410 IU mg^{-1}) from yeast was obtained from Sigma Chemical Co., Ltd.

2.5 SUBSTRATES AND COENZYMES

2.5.1 Hydrogen Peroxide

30% (w/v) H_2O_2 was obtained from BDH Chemicals Ltd. 100mM stock

solutions of this reagent were found to be stable for at least three weeks when stored at 4°C in a dark bottle. Immediately prior to use the molarity of these solutions was checked by titration against a primary standard of potassium permanganate in sulphuric acid (27).

2.5.2 Glucose

Solutions of Analar grade glucose (BDH Chemicals Ltd.) were made up in distilled water at least 24 h before use to ensure complete mutarotation. Such solutions were stored at 4°C and used within 3 days of their preparation.

2.5.3 2,2'Azino-di-(3-ethyl-benzthiazoline sulfonic acid (6)) (ABTS)

ABTS was obtained from Boehringer Corporation London Ltd., and stored dessicated over silica gel at 4°C. Solutions of this reagent were made up in the appropriate buffers and used within 6 h of their preparation.

2.5.4 Acetaldehyde

Acetaldehyde was obtained from May and Baker Ltd., Dagenham, Essex, U.K. This chemical was stored at 4°C and redistilled immediately prior to use. Standard solutions were stored on ice and used within 3 h of their preparation. The concentration of such solutions was determined spectrophotometrically by measuring the oxidation of NADH in the presence of yeast alcohol dehydrogenase, according to the method of Bergmeyer (28).

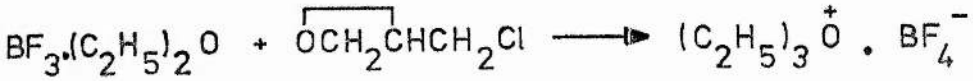
2.5.6 NAD^+

Grade III NAD^+ was obtained from Sigma Chemical Co., and stored dessicated at 4°C. Solutions of this coenzyme were always used within 12 h of their preparation.

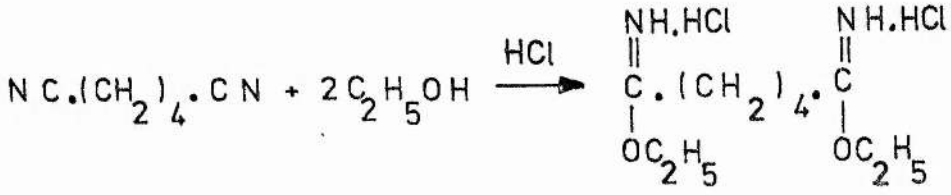
2.5.7 Serum

Bovine whole-blood was collected in heparinised containers in order to prevent any coagulation of the blood. The erythrocytes were centrifuged in an MSE 6L centrifuge (MSE Instruments Ltd., Crawley, Sussex, England, U.K.) for 1 h at 2,000 r.p.m. and a temperature of 7°C. The straw-coloured liquid was collected and stored at -20°C. Immediately prior to use it was quickly thawed and any precipitate that had formed was removed by centrifugation using a bench-centrifuge.

CHAPTER 3. METHODS



(a)



(b)

Figs 1(a) and 1(b) . The synthesis of (a) triethyloxonium tetrafluoroborate ; and (b) diethyladipimidate.

3.1 SYNTHESIS

3.1.1 Triethyloxonium Tetrafluoroborate

Triethyloxonium tetrafluoroborate (TOTFB) was prepared freshly when required by the method of Meerwein (29). 12.5 g of 1-chloro-2 3-epoxypropane was added to a refluxing solution of 25 g borontrifluoride diethyletherate in 250 ml of dry diethyl ether, vigorously stirred on a magnetic stirrer. When all the former reagent had been added, the mixture was refluxed for a further 60 min and then allowed to cool to room temperature with continuous stirring. The precipitated TOTFB was then washed twice with dry ether and stored as a 10% (w/v) solution in dichloromethane. This reaction is shown schematically in Fig 1a.

3.1.2 Diethyl Adipimidate

Diethyl adipimidate was prepared according to the method of Pinner (30). 21.6 g of adiponitrile and 18.6 g of dry ethanol were dissolved in the minimum quantity of dry dioxan. The solution was incubated in an ice water bath in order to maintain the temperature below 5°C. The reaction mixture was then gassed with dry HCl, until 20 g had been dissolved in the dioxan. This was sufficient to provide a 20% excess over the stoichiometric amount required. The reaction mixture was then maintained at 4°C for 30 h, after which, the diethyl adipimidate was precipitated by the addition of dry ether. The precipitated diethyl adipimidate was then washed twice with ether. In the final wash the ether was completely removed by evaporation under vacuum. The dry solid was stored dessicated under vacuum at room temperature. The reaction is shown in Fig 1b.

PREPARATION OF NYLON TUBE IMMOBILISED ENZYMES

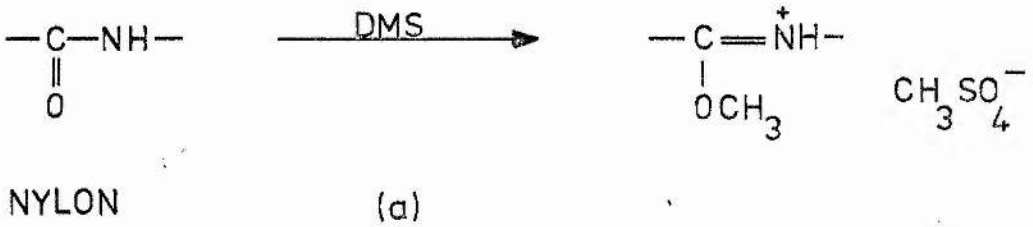
3.2. GENERAL CHEMISTRY OF ATTACHMENT

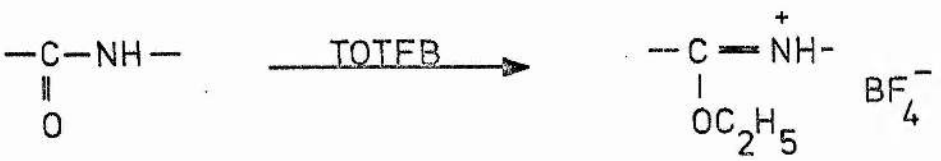
Most nylons are of high molecular weight and consequently contain few reactive end groups. Therefore the covalent attachment of enzymes to nylon must initially entail its modification so that active centres are generated. In this work the necessary modification is achieved through the formation of imidate salts from the reaction of the secondary amide linkages of nylon with powerful alkylating reagents such as dimethyl sulphate and triethyloxonium tetrafluoroborate. Nylon-tube treated in this manner is referred to as O-alkylated nylon-tube. Imidate salts thus formed will react with nucleophiles such as amines and acid hydrazides, yielding the corresponding amidines and amidrazones respectively. The O-alkylated nylon-tubes reacted in this manner with amines, acid hydrazides and egg albumen (otherwise called spacers) are referred to as amine-, hydrazide-, and protein-substituted nylon-tubes respectively.

The free amine or hydrazide functions on the substituted nylon-tubes can be covalently linked to the appropriate enzyme by using bifunctional cross-linking reagents such as glutaraldehyde or diethyl adipimidate. Nylon-tubes prepared for the attachment of the enzyme in this manner are referred to as glutaraldehyde- and diethyl adipimidate-reactivated nylon-tubes respectively.

In the case of hydrazide substituted nylon-tubes, conversion of the hydrazide group to an azide function by nitrous acid enables the enzyme to be directly immobilised to the substituted nylon-tube without the use of cross-linking reagents. Derivatives prepared in this manner are referred to as azide-reactivated nylon-tubes.

Nylon-tube immobilized enzyme tubes are sometimes referred to as enzyme tubes.





Figs 2(a) and 2(b). O-alkylation of nylon-tube with (a) dimethyl sulphate, and (b) triethyloxonium tetrafluoroborate.

The reaction sequences employed in the preparation of nylon-tube immobilised enzymes are schematically described in Figs 2-4.

Unless otherwise specified the nylon-tube immobilised enzymes were prepared by TOTFB alkylation and glutaraldehyde reactivation of the nylon tubes.

3.2.1.1 O-Alkylation of Nylon-tube with Dimethyl Sulphate

3m lengths of nylon-tube were filled with dimethyl sulphate, their ends firmly sealed, and immersed in a boiling water bath for 6 min. Immediately thereafter, the tubes were plunged into an ice-water bath in order to arrest the alkylation. Subsequently, the dimethyl sulphate was pumped out of the tubes, which were then washed by perfusion with 50 ml methanol for 2 min at 4°C. The O-alkylated nylon-tubes thus prepared were then immediately filled with a solution of the appropriate spacer compound as described below. The reaction is schematically represented in Fig 2a.

3.2.1.2 O-Alkylation of Nylon-Tube with TOTFB

3 m lengths of nylon-tube were filled with a 10% (w/v) solution of TOTFB in dichloromethane, their ends firmly sealed, and incubated at room temperature for 12 min. The O-alkylation was terminated by perfusing the tubes with dichloromethane for 2 min. Immediately thereafter the O-alkylated nylon-tubes were incubated with a solution of the appropriate spacer compound as described below. The reaction is schematically represented in Fig 2b.

3.2.2.1 Preparation of Amine-Substituted Nylon-Tube

Amine-substituted nylon-tubes were prepared by filling the

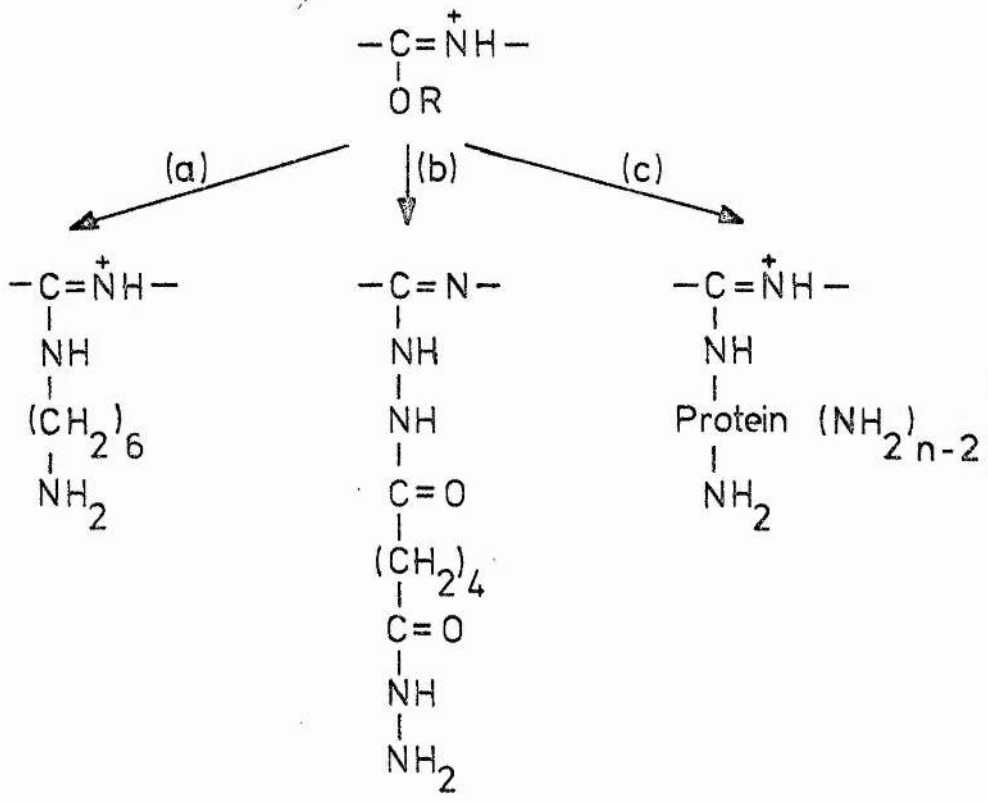


Fig 3. The substitution of O-alkylated nylon-tube with (a) 1,6-diaminohexane, (b) adipic acid dihydrazide, and (c) inert protein.

O-alkylated nylon-tubes with a solution of either 1.0 M- 1, 6-diamino-hexane, or 1.0 M- 1, 2-diaminoethane in methanol. Alternatively, a 0.5M aqueous solution of the diamine was titrated to pH 9.0, and used in this step. In all cases thereafter, the tubes were sealed at both ends and incubated at room temperature for at least 1 h. Excess unreacted amine was then removed by perfusion with water for 2 h. Amine-substituted nylon-tube prepared in this way could be stored for 3 weeks without any detectable deterioration. The preparation of these derivatives is schematically represented in Fig 3.

3.2.2.2. Preparation of Protein-Substituted nylon-tube

Protein-substituted nylon-tubes were prepared by filling the freshly prepared O-alkylated nylon-tubes with either 1.0% (w/v) egg albumen, in 0.2M-borate, pH 9.0, or 1.0% (w/v) methylated bovine serum albumen in methanol. In both cases the ends were sealed and the tubes incubated at room temperature for at least 1 h. Thereafter, excess protein was removed by perfusion with water for 24 h. These protein-substituted nylon-tubes could be stored at room temperature for 3 weeks without any detectable deterioration. The preparation of these derivatives is schematically represented in Fig 3.

For the preparation of denatured protein-substituted nylon-tube, the egg albumen was attached as described above and then denatured by perfusion with a solution of 8.0M-urea in distilled water for 2 h at a flow rate of 2 ml min^{-1}. The tube then washed free of urea by its perfusion with 0.5M-NaCl at a flow rate of 4 ml min^{-1} for 2 h.

3.2.2.3 Preparation of Hydrazide-Substituted Nylon-Tube

Hydrazide-substituted nylon-tubes were prepared by filling the

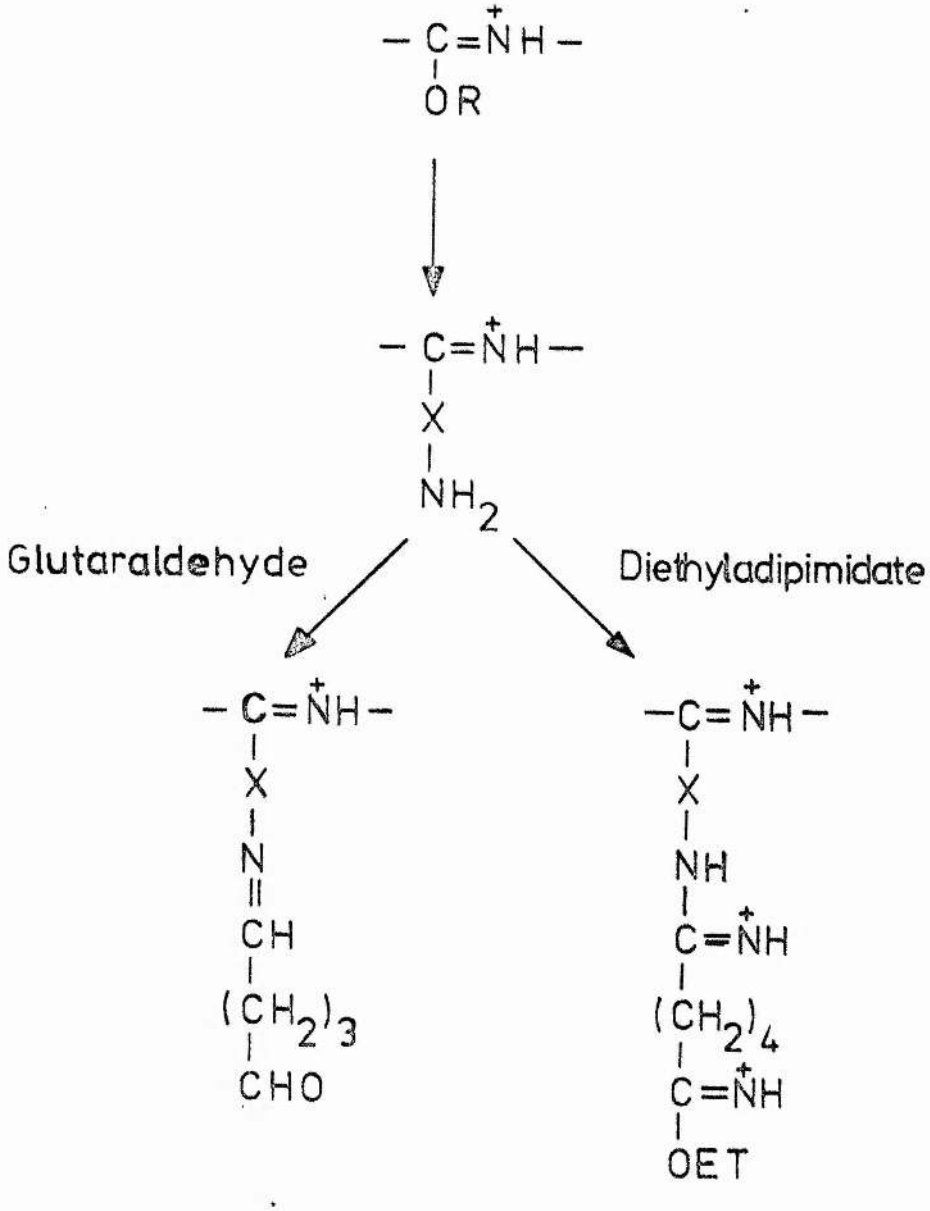


Fig 4. Reactivation of substituted nylon-tube with glutaraldehyde and diethyladipimidate.

freshly prepared O-alkylated nylon-tubes with a 4 mg ml^{-1} solution of either adipic or succinic acid dihydrazide in formamide. The ends were sealed and the tubes incubated at room temperature for at least 1 h. Thereafter, excess hydrazide was removed by perfusion with water for 24 h. The hydrazide substituted nylon-tubes could be stored for 3 weeks without any detectable deterioration.

Hydrazine-substituted nylon-tubes were prepared by filling the O-alkylated nylon-tubes with a 3.0M solution of hydrazine hydrate in methanol. The incubation and removal of excess hydrazine was as described above.

The preparation of both hydrazide- and hydrazine- substituted nylon-tubes is schematically represented in Fig 3.

3.2.3. Reactivation of Substituted Nylon-Tube

3.2.3.1 Reactivation with Glutaraldehyde

Amine-, protein-, and hydrazide-substituted nylon-tubes were reactivated by their perfusion in a close-loop system with a 5% (w/v) solution of glutaraldehyde in 0.2M-borate, pH 8.5, for 15 min at a flow rate of 5 ml min^{-1} and a temperature of 20°C. Thereafter, the tubes were washed free of unreacted glutaraldehyde by their perfusion with 0.2M-phosphate, pH 7.8, for 5 min at a flow rate of 5 ml min^{-1}, and immediately incubated with the appropriate enzyme solution. The reaction is schematically represented in Fig 4.

3.2.3.2 Reactivation with Diethyl Adipimidate

Amine-, protein-, and hydrazide-substituted nylon-tubes were first dried by perfusion with absolute ethanol for 10 min. The tubes were then reactivated by their perfusion at a flow rate of 3 ml min^{-1} in a closed

loop system with a solution containing 3.0ml absolute ethanol, 2.0 ml N-ethyl morpholine, and 100 mg diethyl adipimidate. After 2 h, unreacted diethyl adipimidate was washed free from the tube by perfusion with 50 ml absolute alcohol at a flow rate of 5 ml min^{-1}. Immediately thereafter, the appropriate enzyme solution was drawn into the tube. The reaction is schematically represented in Fig 4.

3.2.3.3 Preparation of Azide-Activated Nylon-Tube

100 ml of 0.1 M-HCl was perfused from a reservoir through succinic acid dihydrazide-substituted nylon-tube in a closed loop at a flow rate of 3 ml min^{-1}. The temperature was maintained throughout at 0°C by means of a salt-ice bath. 1.5 g of sodium nitrite in 10 ml ice-cold water was added dropwise to the reservoir over a period of 10 min. After 30 min the tube was washed free of acid by perfusion with 30 ml ice-cold 0.2M-phosphate, pH 7.8, at a flow rate of 10 ml min^{-1}. The tube was then immediately filled with the appropriate enzyme solution.

3.2.4 Coupling of Enzymes to Reactivated Nylon-Tube

Immediately after the preparation of reactivated nylon-tube, a solution of the appropriate enzyme was drawn into the tube by means of a syringe. The ends were clamped and the tube was incubated at room temperature for 3 h. The tube was then perfused with 15 ml 0.2M-phosphate, pH 7.8, and the washings collected for assay. Thereafter, excess unbound enzyme was washed off the support by its perfusion with 500 ml 0.5 M-NaCl at a flow rate of 5 ml min^{-1}. All nylon-tube immobilised enzyme derivatives were stored at 4°C, filled with 0.05M-phosphate, pH 6.9.

3.3 Estimation of Bound Enzyme

The amount of enzyme immobilised on the inside surface of nylon-tube was estimated in the following manner. After the coupling reaction, the enzyme solution was withdrawn from the tube, which was then perfused with 15 ml of 0.2 M-phosphate, pH 7.8. The enzyme coupling solution and the washings were pooled and the total number of enzyme units therein estimated. An aliquot of the enzyme coupling solution was retained prior to its incubation with the tube and the number of enzyme units in the whole volume thereby estimated. Comparison of the two enzyme activities yielded the amount of enzyme taken up from the solution by the immobilisation reaction. A control was employed in which an aliquot of the enzyme solution was assayed before and after being subjected to the conditions used in the enzyme coupling reaction. In this way, any decrease in activity due to enzyme denaturation rather than immobilisation could be taken into account.

3.4. DETERMINATION OF SOLUBLE ENZYME ACTIVITY

3.4.1 Catalase

The catalatic activity of catalase was measured at 25°C by recording the disappearance of H_2O_2 spectrophotometrically at 240 nm, according to the method of Bergmeyer (31). All assays were performed in a reaction volume of 3 ml containing 15 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9. In each case the reaction was initiated by the addition of 10 µl of the enzyme solution. One unit of catalase activity is defined as the amount of catalase required to decompose one µmole of H_2O_2 per minute at pH 6.9 at 25°, while the H_2O_2 concentration falls from 10.3 to 9.2 µmoles ml^{-1} of reaction mixture.

3.4.2. Glucose Oxidase

Glucose oxidase activity was measured at 25°C by recording the disappearance of dissolved oxygen with a Yellow Springs Model 53 Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). All assays were performed in a 5 ml reaction volume containing 4 mM-glucose in 0.05 M-acetate, pH 5.6. In each case the reaction was initiated by the addition of 10 ul of the enzyme solution. The activity is expressed in terms of μmol O_2 utilised per min.

3.4.3 Peroxidase

Peroxidase activity was measured at 25°C by measuring the oxidation of ABTS spectrophotometrically at 610 nm. All assays were performed in a 3 ml reaction volume containing 1.0 mM-H_2O_2 and 5.0 mM-ABTS in 0.05 M-phosphate, pH 6.9. In each case the reaction was initiated by the addition of 10 μl of the enzyme solution. The activity is expressed in terms of μmol ABTS oxidised per min.

3.4.4 Aldehyde Dehydrogenase

Aldehyde dehydrogenase activity was determined spectrophotometrically by measuring the production of NADH at 340 nm in the manner of Bergmeyer (32). All assays were performed at 25°C in a 3 ml reaction volume containing 1.0 mM-acetaldehyde; 0.2 mM-NAD^+; 0.5 mM-dithioerythritol; 0.2 M-KCl; 5 mM-EDTA; and 0.1 M-tris, pH 8.0. In each case the reaction was initiated by the addition of 10 μl of the enzyme solution. An $E^{1\,cm}_{340}$ for NADH of 6.22×10^6 $cm^2 Mol^{-1}$ was used throughout. The activity is expressed in terms of μmol NADH produced per min.

3.5 ESTIMATION OF IMMOBILISED ENZYME ACTIVITY

The activity of nylon-tube immobilised enzymes was determined in one

of two ways. In one method the extent of conversion of substrate in a single pass of the enzyme tube was estimated, and in the other the recirculation assay system proposed by Ford and Lambert (33) was employed.

In the following mathematical treatments, any effects arising from diffusion of substrate, or product from the enzyme are neglected in order to simplify the theor etical analyses. Although it has been shown that diffusional effects can occur in open-tubular heterogeneous reactors (34,35), it is not unreasonable to assume that such effects occurring in the assay systems described here will be of little importance due to the following considerations.

From the nature of the enzyme immobilisation process used in this work, the enzyme will be mainly located at the surface of the support. Therefore any such effects arising will be due to film-diffusion and not pore diffusion. The recirculation assay procedure requires high flow rates, and as film diffusion is related inversely to the liquid flow, any effects upon the system will be minimised. In the case of the single pass assay, the enzyme tube was coiled into the shape of a tight helix, and the liquid stream segmented with air. Both these precautions serve to minimise any effects of film diffusion; the helical conformation improves mixing characteristics and the air segment continually scours the nylon surface.

3.5.1 Recirculation Assay System

The recirculation assay system is shown schematically in Fig 5. The substrate was maintained at 25°C in the reservoir, vigorous mixing was achieved by means of a magnetic stirrer. The substrate was pumped through the closed loop containing the enzyme derivative under assay at a flow rate of 35 ml min^{-1} by means of a peristaltic pump (Watson-

Marlow Ltd., Falmouth, Cornwall, England, U.K.).

Two methods were employed to monitor the reaction. Where the emergence of product or depletion of substrate could be continuously followed spectrophotometrically, a 1 cm light path flow cell was incorporated into the system at point X and the change in extinction followed on a Beckmann DB spectrophotometer. Where this method was inapplicable, aliquots were withdrawn from the reservoir at various times during the assay and analysed for the appearance of product or the disappearance of substrate.

According to Ford & Lambert, consideration of a material balance across the entire recirculation reactor yields the following equation:-

$$r = \frac{-dS}{dt} \cdot V_f / W \qquad \text{.....(1)}$$

where r = reaction rate of immobilised enzyme (mol min^{-1} mg^{-1})

$\frac{-dS}{dt}$ = observed rate of decrease of substrate concentration (mol min^{-1} cm^{-3})

V_f = total fluid volume of system (cm^{-3})

W = amount of immobilised enzyme (mg)

Rearrangement of eqn 1 gives:-

$$rW = \frac{-dS}{dt} \cdot V_f$$

or

$$C = \frac{-dS}{dt} \cdot V_f \qquad \text{.....(2)}$$

where C = amount of substrate converted by the immobilised enzyme in unit time (mol min^{-1})

For an enzyme catalysed reaction, C is given by the eqn :-

$$C = v \cdot V_e \qquad \text{.....(3)}$$

Where v = velocity of the reaction (mol min^{-1} cm^{-3})

V_e = volume in which the enzyme is dispersed (cm^3)

In the case of a nylon-tube immobilised enzyme, V_e corresponds to the volume of the immobilised enzyme tube. The reaction velocity, v , of an enzyme-catalysed reaction is described by the Michaelis-Menten

equation:-

$$v = \frac{k_s \cdot E_c \cdot S}{S + K} \qquad \text{.....(4)}$$

Where k_s = specific activity of enzyme (mol min^{-1} mg^{-1})

E_c = volumetric concentration of the enzyme (mg cm^{-3})

S = substrate concentration (mol cm^{-3})

K = Michaelis constant (mol cm^{-3})

From equations (2), (3) and (4)

$$\frac{k_s \cdot E_c \cdot S \cdot Ve}{S + K} = \frac{-dS}{dt} \cdot V_f$$

Thus,

$$\frac{k_s \cdot E \cdot S}{S + K} = \cdot \frac{-dS}{dt} \cdot V_f$$

Where E = total amount of enzyme in the system (mg)

Consider an immobilised enzyme of a tubular form with the following characteristics:-

L = length of tube (cm)

r = radius of tube (cm)

E_a = surface concentration of enzyme on the inner surface of the tube (mg cm^{-2})

k_t = tube-specific activity (mol min^{-1} cm^{-1})

In this case, the total amount of enzyme in the tube, E, is given by,

$$E = E_a \cdot 2\pi \cdot r \cdot L \qquad \text{.....(6)}$$

The total amount of activity displayed by the tube, A, is given by :-

$$A = k_t \cdot L$$

However,

$$k_s = \frac{A}{E_a}$$

and therefore,

$$k_s = \frac{k_t \cdot L}{E_a \cdot 2\pi \cdot r \cdot L} = \frac{k_t}{E_a \cdot 2\pi \cdot r} \qquad \text{.....(7)}$$

From eqns (5), (6) and (7),

$$\frac{k_t}{E_a \cdot 2\pi \cdot r} \cdot \frac{E_a \cdot 2\pi \cdot r \cdot L \cdot S}{S + K} = \frac{-dS}{dt} \cdot V_f$$

or,

$$\frac{k_t \cdot L \cdot S}{S + K} = \frac{-dS}{dt} \cdot V_f \quad \text{.....(8)}$$

For most enzymes it is possible to arrange the experimental conditions so that $S \gg K$ and the reaction is zero order with respect to substrate.

In this case,

$$k_t \cdot L = \frac{-dS}{dt} \cdot V_f$$

and so,

$$k_t = \frac{-dS}{dt} \cdot V_f \cdot \frac{1}{L} \quad \text{.....(9)}$$

Equation (9) permits the evaluation of the tube specific activity, k_t, by measurement of the rate of utilisation of the substrate.

For some enzymes, such as catalase, kinetic measurements are made under conditions in which $S \ll K$. In this case the activity of the tube-immobilised enzyme is described by the equation:-

$$\frac{k_t \cdot L \cdot S}{K} = \frac{-dS}{dt} \cdot V_f \quad \text{.....(10)}$$

Equation (10) describes a first-order reaction and consequently measurement of the term $\frac{-dS}{dt}$ is difficult since the progress curve of the reaction does not display any linear regions. In this case, k_t is evaluated by using the integrated form of equation (10).

Separating the variables and integrating yields:-

$$\ln \frac{S_o}{S} = \frac{k_t \cdot L \cdot t}{V_f \cdot K} \quad \text{.....(11)}$$

Where t = elapsed reaction time (min)

S_o = substrate concentration at time t = zero (mol cm^{-3})

S = substrate concentration at time t (mol cm^{-3})

Equation (11) permits the calculation of the parameter $\frac{k_t}{K}$, which has the units of $cm^2\ min^{-1}$ and is used as a measure of the catalytic activity of the nylon-tube immobilised enzyme.

In all assays a limit of 2% conversion of substrate per pass of the enzyme tube was rigorously maintained, in order to minimise errors arising through rapid conversion of substrate. This control was effected by careful choice of the length of enzyme tube submitted for assay. In practise, lengths of between 15 cm and 50 cm were employed, depending upon the activity of the derivatives.

3.5.2. Single Pass Assay

Immobilised enzyme activity was also measured by means of a single pass assay system in the following manner. The substrate in the appropriate buffer was perfused through the enzyme derivative at a flow rate that ensured conversion of between 10 - 90% of the substrate. After perfusion of the tube for 5 min, a sample of the tube effluent was collected and analysed for either the formation of product or the utilisation of substrate.

In the following mathematical treatment of the system, the effect of diffusion has been ignored for the reasons stated in the previous section.

The integrated rate equation states that:-

$$(S_o - S) + K \cdot \ln (S_o / S) = V.t \qquad \ldots\ldots\ldots\ldots\ldots\ldots (12)$$

where S_o = Initial substrate concentration (mol cm^{-3})

S = Substrate concentration at time t (mol cm^{-3})

K = Michaelis constant (mol cm^{-3})

V = Reaction velocity (mol min^{-1} cm^{-3})

t = Time of reaction (min)

From section 3.5.1

$$V = k_s \cdot E_c \qquad \ldots\ldots\ldots\ldots\ldots\ldots (13)$$

and,

$$E_c = \frac{E_a \cdot 2\pi \cdot r \cdot l}{\pi \cdot r \cdot L} = \frac{2E_a}{r} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (14)$$

and,

$$k_s = \frac{k_t \cdot L}{2\pi \cdot r \cdot L \cdot E_a} = \frac{k_t}{2\pi \cdot r \cdot E_a} \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

k_t = Tube specific activity (mol min^{-1} cm^{-1})

where k_s = Specific activity of immobilised enzyme (mol min^{-1} mg^{-1})

E_c = Volumetric concentration of the enzyme (mg cm^{-3})

E_a = Surface concentration of enzyme in the inner surface of the tube (mg cm^{-3})

r = Radius of enzyme tube (cm)

L = Length of enzyme tube (cm)

Thus $$V = k_s \cdot E_c = \frac{2E_a}{r} \cdot \frac{k_t}{2\pi r \cdot E_a} = \frac{k_t}{\pi \cdot r^2} \quad \ldots\ldots\ldots\ldots (15)$$

In the case of a nylon-tube immobilised enzyme, t, the time of contact of the enzyme with its substrates, is the resonance time of the substrate in the tube

Therefore,

$$t = \frac{r^2 \cdot \pi \cdot L}{Q}$$

where Q = volumetric flow rate of substrate through tube (cm^3 min^{-1})

and,

$$V \cdot t = \frac{r^2 \cdot \pi \cdot L}{Q} \cdot \frac{k_t}{\pi \cdot r^2} = \frac{k_t \cdot L}{Q} \quad \ldots\ldots\ldots\ldots\ldots (16)$$

Now,

$$(S_o - S) = P$$

where P = concentration of product (mol cm^{-3})

therefore, substituting in eqn 12

$$P + K \cdot \ln S_o / S_o - P = \frac{k_t \cdot L}{Q}$$

Or

$$P = \frac{k_t \cdot L}{Q} - K \cdot \ln (S_o / S_o - P) \quad \ldots\ldots\ldots\ldots\ldots (17)$$

When considering the single pass assay system as a means of monitoring nylon-tube immobilised catalase stability, rearrangement of Eqn 17 yields:-

$$\text{Ln}\frac{S_o}{S_o - P} = \frac{K_t \cdot L}{Q \cdot K} - \frac{(S_o - S)}{K} \quad \ldots\ldots\ldots\ldots\ldots(18)$$

But $K \gg S_o$ as catalase obeys first order kinetics. Therefore the term $\frac{S_o - S}{K}$ becomes insignificant.

$$\text{Ln}\frac{S_o}{S_o - P} = \frac{k_t \cdot L}{K \cdot Q}$$

or,

$$\text{Ln}\frac{S_o}{S} = \frac{k_t \cdot L}{K \cdot Q} \quad \ldots\ldots\ldots\ldots\ldots(19)$$

Thus $\text{Ln}\frac{S_o}{S}$ is directly proportional to the tube specific activity (k_t) of the immobilised catalase derivative under the defined assay conditions. In practise, when monitoring the stability of nylon-tube immobilised catalase to H_2O_2 at pH 6.9 and 25°C, the flow-rate of the substrate and the length of the derivative were always maintained at 2.9 ml min^{-1} and 1.5 m respectively. In this manner the stability of various derivatives could be directly compared.

3.5.4 Catalatic Activity of Nylon-Tube immobilised Catalase Using the Recirculation Assay

The reservoir contained 100 ml 1.0 mM-H_2O_2 in 0.1 M-phosphate, pH 6.8. At intervals of 3 min, 0.2 ml aliquots were withdrawn from the reservoir and assayed for H_2O_2 depletion in the following manner. The aliquots were placed in 1.7 ml of 4 mM-ABTS in 0.05 M-phosphate, pH 6.9, containing 50 µg peroxidase. The mixture was incubated at 25°C for 10 min to allow the reaction to go to completion, whereupon its extinction, at 610 nm, was read. Standard curves were compiled by submitting 0.2 ml aliquots of standardised H_2O_2 to the same treatment. The depletion of H_2O_2 in the assay was then followed by comparison with the standard curve.

3.5.5 Peroxidatic Activity of Nylon-Tube Immobilised Catalase Using the Recirculation Assay

The reservoir contained 100 ml 1 mM-H_2O_2, and 2.5 M-methanol in 0.1 M-phosphate, pH 6.9. At intervals of 2 min, 0.2 ml aliquots were withdrawn from the reservoir and assayed for formaldehyde using the Hantzsch reaction (25.). The aliquots were added to a mixture containing 50 μmoles acetylacetone, 800 μmoles methanol, 300 μmoles $NH_4H_2PO_4$, pH 6.0, in a final volume of 0.5 ml. Thereafter the mixture was incubated at 37°C for 20 min and its absorbance at 412 nm measured. Standard curves were compiled by submitting 0.2 ml aliquots of standardised HCHO to the same treatment. The emergence of HCHO was then followed by comparison with the standard curve.

3.5.6 Nylon-Tube Immobilised Glucose Oxidase Activity Using the Recirculation Assay

The reservoir contained 100 ml 0.3 mM-glucose in 0.05 M-phosphate, pH 6.8, maintained at 25°C. At 5 min intervals, 1.0 ml aliquots were withdrawn and assayed for glucose depletion using the coupled reaction of hexokinase and glucose-6-phosphate dehydrogenase. The aliquots were added to a mixture containing 100 mM-tris, pH 7.8, 3 mM-$MgCl_2$, 0.2 mM-ATP, 0.2 mM-NADP, 10 μg hexokinase and 10 μg glucose-6-phosphate dehydrogenase in a final volume of 3 ml. The mixture was incubated for 40 min at 25°C to allow the reaction to go to completion, whereupon the extinction at 340 nm was measured. 1.0 ml aliquots of standardised glucose solutions were also submitted to the above procedure and standard curves compiled. From comparison with these, the rate of depletion of glucose was monitored.

3.5.7 Nylon-Tube Co-immobilised Glucose Oxidase and Catalase Using the Recirculation Assay

The reservoir contained 100 ml 0.3 mM-glucose and 2.5 M-methanol

in 0.05 M-phosphate, pH 6.9. The coupled reaction was followed by the withdrawal of aliquots from the reservoir and their analysis for formaldehyde as described in section 3.5.5.

3.5.8 Nylon-Tube Immobilised Aldehyde Dehydrogenase Using the Recirculation Assay

The reservoir contained 20 μmoles acetaldehyde, 4 μmoles NAD^+, 10 μmoles dithioerythritol, 100 μmoles EDTA, 4 mmoles KCl, and 2 mmoles tris, pH 8.5 in a final volume of 20 ml. A flow-cell was encorporated into the recirculation assay system at point X and the production of NADH monitored spectrophotometrically at 340 nm. An $e^{1\ cm}_{340}$ of 6.22×10^4 for NADH was used throughout.

3.6 AUTOANALYSIS

Autoanalysis systems were constructed from standard AA 1 Technicon modules. Extinction were measured by passing the de-bubbled stream through a 1 cm light-path flow cell contained in a Beckmann DB spectrophotometer.

Reagent dilutions in the systems were periodically checked to ensure the uniformity of the pumping rates. This was done by sampling an NADH solution of known extinction and subsequently measuring the extinction of the diluted sample. Comparison of the two measurements enabled the actual dilution of the system to be calculated and compared with the nominal pump-rates. Volumetric flow rates were estimated by the measurement of the quantity of liquid collected over a 10 min period. The following abbreviations are used in the schematic representation of the flow systems:-

SMC = Short mixing coil

DC = Delay coil

DB = De-bubbler

D = Dialyser

W = Waste

ET = Enzyme Tube

O = Sample line

SPEC = Spectrophotometer

3.6.1 Stability of Nylon-Tube Immobilised Catalase

The stability of nylon-tube immobilised catalase derivatives to continuous exposure to 10 mM-H_2O_2 was studied at pH 6.9 and 25°C. A 1.5 m length of the derivative was incorporated at position ET in the flow system described in Fig 6. The activity of the tube was monitored by the measurement of the unconverted H_2O_2 issuing from the tube. This was done by the addition of acid-KI to the tube effluent and measurement of the extinction at 349 nm. The system was intermittently calibrated by the removal of the enzyme tube and the analysis of standard H_2O_2 solutions.

3.6.2 The effect of pH on the Activity of Nylon-Tube Immobilised Glucose Oxidase

The effect of pH on the activity of nylon-tube immobilised glucose oxidase was investigated by its insertion at position ET in the flow system described in Fig 7. 4 mM solutions of glucose were sampled and the H_2O_2 produced from the glucose oxidase reaction was measured by means of the acid-KI technique. In practise, the length of derivative was chosen such that neither the depletion of dissolved oxygen nor glucose exceeded 15%. In most cases, a 50 cm length was found to satisfy these conditions. The system was calibrated by the removal of the enzyme tube

and the analysis of standard H_2O_2 solutions.

3.6.3 The Effect of pH on the Activity of Nylon-tube Co-immobilised Glucose Oxidase and Catalase

The effect of pH on the activity of HMDA-substituted nylon-tube co-immobilised glucose oxidase and catalase was investigated by means of its insertion at position ET in the flow system described in Fig 8a. In this case, the H_2O_2 produced from the glucose oxidase reaction was monitored by its conversion to HCHO using the peroxidatic reaction of catalase.

The formaldehyde production was measured by means of the Hantzsch reaction (25). The whole reaction sequence is schematically represented in Fig 9. Standard solutions of HCHO were prepared immediately prior to use and stored on ice to prevent any anomolies arising from the low boiling point of HCHO. The system was calibrated by the removal of the enzyme tube and the analysis of the standard HCHO solutions.

$$\text{Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconolactone} + H_2O_2 \xrightarrow[\text{methanol}]{\text{catalase}} HCHO + 2H_2O$$

$$HCHO + NH_4^+ + CH_3CO.CH_2CO.CH_3 =$$

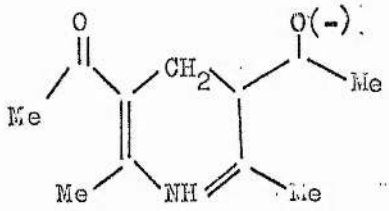


Fig. 9. Analysis of glucose using glucose oxidase, catalase and the the Hantzsch reaction.

3.6.4 The Peroxidation Reaction of Nylon-Tube Immobilised Catalase

The peroxidatic reaction of nylon-tube immobilised catalase was investigated at pH 6.9 and 25°C by insertion of the derivative at

position ET in the flow system described in Fig 8b. The HCHO produced from the reaction of catalase was monitored by means of the Hantzsch reaction as described in section 3.6.3. The system was calibrated by the removal of the enzyme tube and the analysis of standard HCHO solutions.

3.6.5. Determination of Glucose using Nylon-Tube Co-immobilised Glucose Oxidase and Catalase

Glucose was determined at pH 6.9 and 25°C by the incorporation of a length of HMDA-substituted nylon-tube co-immobilised glucose oxidase and catalase in the flow system described in Fig 8c. The H_2O_2 produced from the glucose oxidase reaction was monitored by its conversion to HCHO using the peroxidatic activity of catalase. The HCHO produced from the catalase reaction was measured by means of the Hantzsch reaction. The whole reaction sequence is schematically shown in Fig 9. The system was calibrated by the analysis of standard H_2O_2, HCHO and glucose solutions.

3.6.6. Determination of Serum Glucose using Nylon-Tube Co-immobilised Glucose Oxidase and Catalase

Serum glucose was determined at pH 6.9 and 25°C by the incorporation of a length of HMDA-substituted nylon-tube co-immobilised glucose oxidase and catalase at position ET in the flow system described in Fig 10a. The H_2O_2 produced from the glucose oxidase reaction was monitored by its conversion to HCHO using the peroxidatic activity of catalase. The HCHO produced from the catalase reaction was measured by means of the Hantzsch reaction. The whole reaction sequence is schematically represented in Fig 9. The system was calibrated by the analysis of standard H_2O_2, HCHO and aqueous glucose solutions.

3.6.7. Determination of Glucose using Separately Immobilised Derivatives of Glucose Oxidase and Catalase in Series

Glucose was determined at pH 6.9 and 25°C by the incorporation of

lengths of HMDA-substituted nylon-tube immobilised derivatives of glucose oxidase and catalase at positions ET_1 and ET_2 respectively in the flow system described in Fig 8d. The H_2O_2 produced from the glucose oxidase reaction was monitored by its conversion to HCHO using the peroxidatic activity of catalase. The HCHO produced from the reaction of catalase was measured by means of the Hantzsch reaction. The whole reaction sequence is schematically represented in Fig 9. The system was calibrated by analysis of standard H_2O_2, HCHO and glucose solutions.

3.6.8. Determination of Serum Glucose using Separately Immobilised Derivatives of Glucose Oxidase and Catalase

Serum glucose was determined, at pH 6.9 and 25°C by the incorporation of lengths of HMDA-substituted nylon-tube immobilised derivatives of glucose oxidase and catalase at positions ET_1 and ET_2 respectively in the flow system described in Fig 10b. The H_2O_2 produced from the glucose oxidase reaction was monitored by its conversion to HCHO using the peroxidatic activity of catalase. The HCHO produced from the catalase reaction was measured by means of the Hantzsch reaction. The whole reaction sequence is schematically represented in Fig 9. The system was calibrated by the analysis of standard solutions of H_2O_2, HCHO and aqeous glucose.

$$\text{Glucose} + \text{Oxygen} \xrightarrow{\text{glucose oxidase}} \delta\text{-gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{Ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + H_2O$$

$$\text{Acetaldehyde} + NAD^+ \xrightarrow{\text{aldehyde dehydrogenase}} \text{acetate} + NADH$$

Fig 11. Analysis of glucose using glucose oxidase, catalase and and aldehyde dehydrogenase.

3.6.9. Determination of Serum Glucose using Nylon-Tube Immobilised Derivatives of Glucose Oxidase, Catalase and Aldehyde Dehydrogenase in Series

Serum glucose was determined at pH 6.9 and 25°C by the incorporation of nylon-tube derivatives of glucose oxidase, catalase and aldehyde dehydrogenase at positions ET_1, ET_2 and ET_3 in the flow system described in Fig 12.

The H_2O_2 produced from the glucose oxidase reaction was monitored by its conversion to acetaldehyde using the peroxidatic activity of catalase. The acetaldehyde produced by this reaction was in turn monitored by the aldehyde dehydrogenase reaction where it was converted to acetate with the production of NADH. The formation of NADH was measured spectrophotometrically at 340 nm. The whole reaction sequence is schematically represented in Fig 11. The system was calibrated by the analysis of standard solutions of glucose.

3.6.10. Determination of Glucose using Nylon-Tube Co-Immobilised Glucose Oxidase and Catalase in Conjunction with a Flow-Through Oxygen Electrode

Glucose was determined at pH 5.6 and 25°C by the insertion of a HMDA-substituted nylon tube co-immobilised derivative of glucose oxidase and catalase at position ET in the flow system described in Fig 13. The oxygen utilised by the glucose oxidase reaction and not replaced by the catalase reaction was monitored by the inclusion of a flow-through oxygen electrode(36)into the system at position O. The instrument was calibrated with 100% air saturated water by the passage of liquid through the system. Calibration with 0% saturated water was achieved by the addition into the liquid stream of sodium dithionite.

Fig 6. Flow system used for the study of the stability of nylon-tube immobilised catalase.

The composition and flow rates of the various pump tubing lines were as follows:-

LINE	REAGENT	FLOW RATE (ml m
1	0.05 M-phosphate, pH 6.9	2.9
2	Air	0.6
3	136 mM-H_2O_2	0.23
4	Effluent from enzyme tube	0.1
5	0.5 M-NaCl, 1% Tween	2.9
6	Air	1.0
7	3.0 M-HCl	1.0
8	0.3 M-KI	1.0
9	(To Waste)	3.9

The nylon-tube immobilised catalase was inserted at position ET and maintained at 25°C. The 30 turn delay coil was maintained at 37°C. Extinctions were measured at 349 nm.

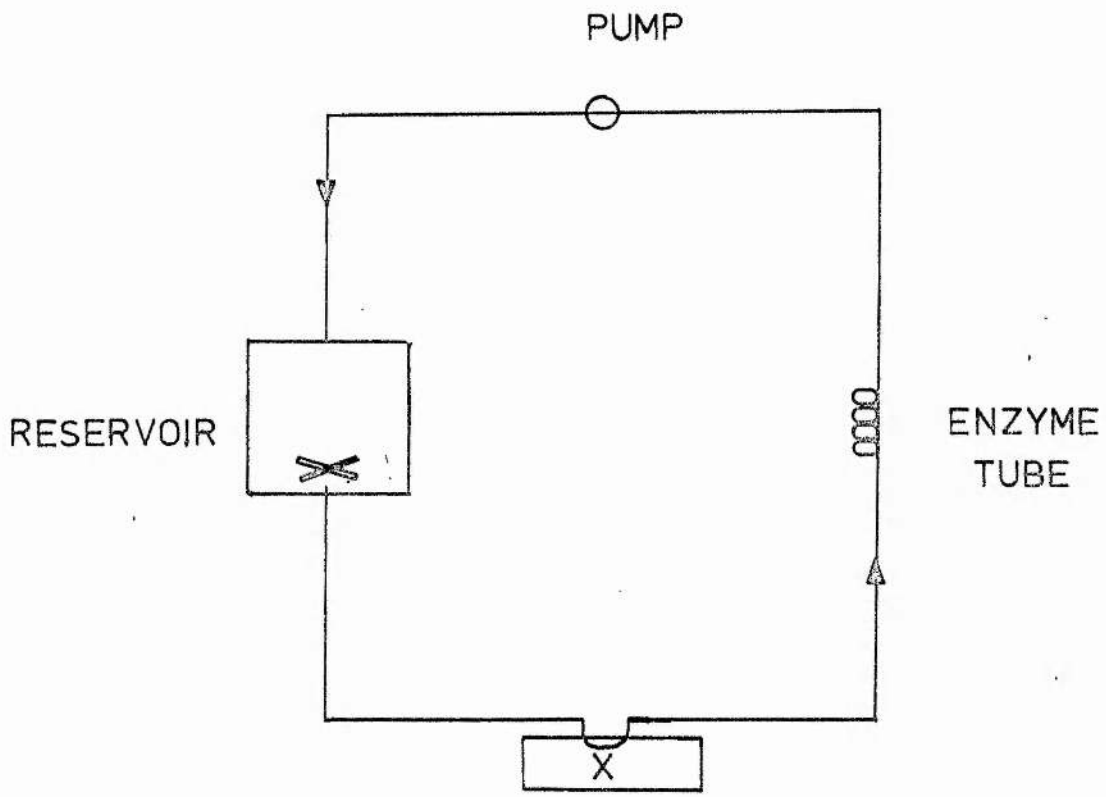


Fig 5. Ford.recirculation assay for the determination of nylon-tube immobilised enzyme activity.

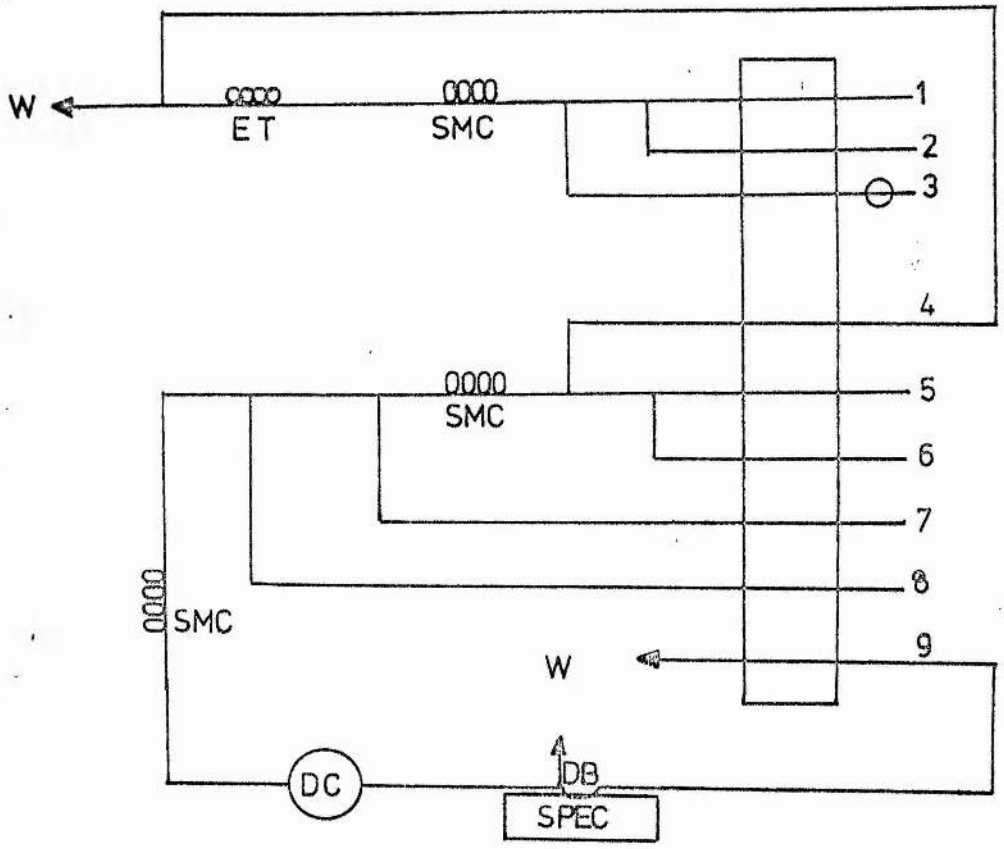

W
ET
SMC
1
2
3
4
SMC
5
6
7
8
SMC
W
9
DC
DB
SPEC

Fig 7. Flow system used for the determination of the effect of pH upon the activity of nylon-tube immobilised glucose oxidase.

The composition and flow rate of the various pump tubing lines were as follows:-

LINE	REAGENT	FLOW RATE (ml m
1	*Reagent E	1.4
2	Air	0.42
3	4 mM-glucose	0.23
4	4 mM-HCl	0.6
5	0.5 M-KI	0.6
6	(To Waste)	2.5

*Reagent E comprised 0.05 M-Acetate or 0.05 M-Phosphate titrated to the appropriate pH.

The nylon-tube immobilised enzyme was inserted at position ET and maintained at 25°C. The 30 turn delay coil was maintained at 37°C. Extinctions were measured at 349 nm. A sample:wash ratio of 2:1 was used throughout and samples were analysed at a rate of 30 per hour.

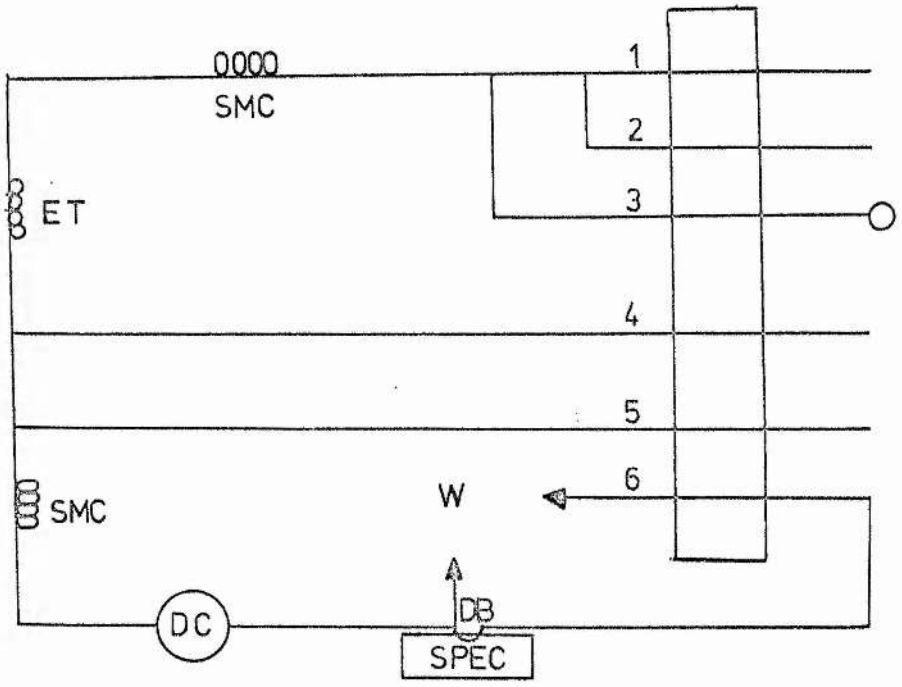

0000
SMC
1
2
3
ET
4
5
SMC
W
6
DC
DB
SPEC

Fig 8a. Flow system used for the determination of the effect of pH on the activity of nylon-tube co-immobilised glucose oxidase and catalase.

The composition and flow rate of the various pump tubing lines were as follows:-

LINE	REAGENT	FLOW RATE (ml min^{-1})
1	* Reagent A	1.4
2	Air	0.6
3	4 mM-glucose	0.23
4	** Reagent B	1.0
5	(To Waste)	2.0

* Reagent A comprised : 2.5 M-methanol, 0.05 M-phosphate or 0.05 M-acetate. titrated to the appropriate pH.

** Reagent B comprised : 0.82 M-acetylacetone, 2.5 M-methanol in 1.0 M-$NH_4H_2PO_4$, pH 6.0.

The co-immobilised enzyme derivatives was inserted at position ET, and was maintained at a temperature of 25°C. The 30 turn delay coil was maintained at 37°C. Extinctions were measured at 412 nm, and a sample: wash ratio of 2:1 was used throughout. Samples were assayed at a rate of 30 per hour.

Fig 8b. Flow system used to study the peroxidatic activity of nylon-tube immobilised catalase.

The composition and flow rates of the various pump tubing lines were as follows:-

LINE	REAGENT	FLOW RATE (ml min^{-1})
1	* Reagent A	1.4
2	Air	0.6
3	H_2O_2	0.1
4	** Reagent B	1.0
5	(To Waste)	2.0

* Reagent A comprised : 2.5 M-methanol in 0.05 M-phosphate, pH 6.9

** Reagent B comprised : 0.82 M-acetylacetone, 2.5 M-methanol in 1.0 M-$NH_4H_2PO_4$, pH 6.0.

The immobilised enzyme derivative was inserted at position ET, and was maintained at a temperature of 25°C. The 30 turn delay coil was maintained at 37°C. Extinctions were measured at 412 nm and a sample:wash ratio of 2:1 was used throughout. Samples were assayed at a rate of 30 per hour.

Fig 8c. Flow system used for the determination of glucose using nylon-tube co-immobilised glucose oxidase and catalase.

The composition and flow rates of the various pump-tubing lines were as follows:-

LINE	REAGENT	FLOW RATE ($ml \cdot min^{-1}$)
1	* Reagent A	1.2
2	Air	0.42
3	Glucose Sample	0.1
4	** Reagent B	0.8
5	(To Waste)	2.0

* Reagent A comprised : 2.5 M-methanol in 0.05 M-phosphate, pH 6.9

** Reagent B comprised : 0.82 M-acetylacetone, 2.5 M-methanol in 1.0 M-$NH_4H_2PO_4$, pH 6.0

The co-immobilised enzyme derivative was inserted at position ET and maintained at 25°C. The 30 turn delay coil was maintained at 37°C. Extinctions were measured at 412 nm and a sample:wash ratio of 2:1 was used throughout. Samples were assayed at a rate of 60 per hour unless otherwise specified.

Fig 8d. Flow system used for the determination of glucose using separately immobilised nylon-tube derivatives of glucose oxidase and catalase in series.

The composition and flow rate of the various pump-tubing lines was as follows:-

LINE	REAGENT	FLOW RATE (ml min^{-1})
1	* Reagent A	1.2
2	Air	0.42
3	Glucose Sample	0.1
4	** Reagent B	0.8
5	(To waste)	2.0

* Reagent A comprised : 2.5 M-methanol in 0.05 M-phosphate, pH 6.9.

** Reagent B comprised : 0.82 M-acetylacetone, 2.5 M-methanol in 1.0 M-$NH_4H_2PO_4$, pH 6.0.

The nylon-tube immobilised derivatives of glucose oxidase and catalase were inserted at positions ET_1, and ET_2 respectively. The immobilised enzymes were maintained at 25°C and the 30 turn delay coil at 37°C. Extinctions were measured at 412 nm and a sample:wash ratio of 2:1 used throughout. Samples were assayed at a rate of 60 per hour unless otherwise specified.

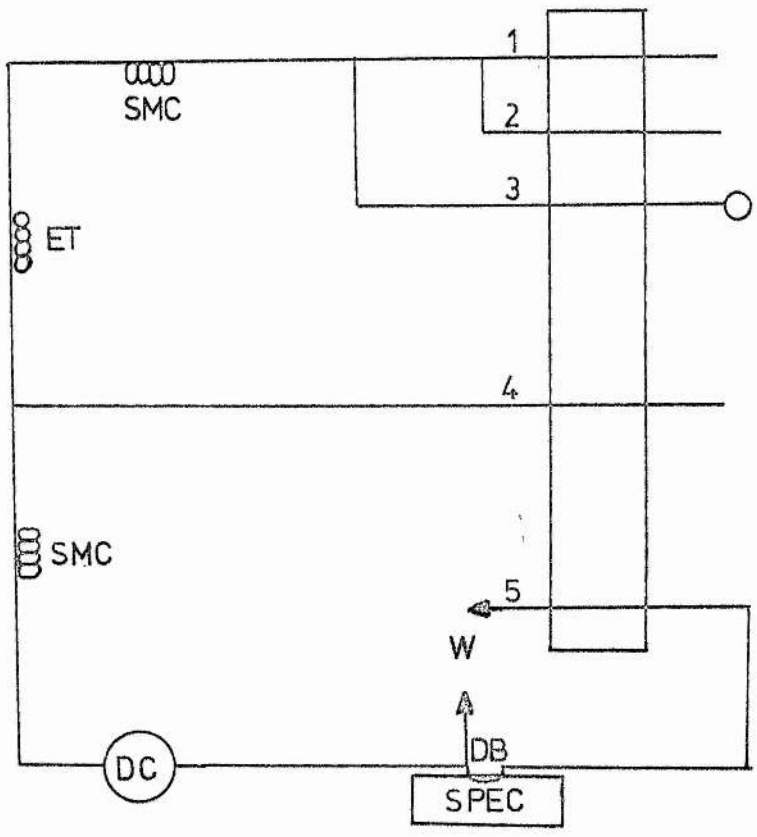


Fig 8(a-d).

Fig 10a. Flow system used for the determination of serum glucose with nylon-tube co-immobilised glucose oxidase and catalase.

The composition and flow rate of the various pump-tubing lines were as follows:-

LINE	REAGENT	FLOW RATE (ml min^{-1})
1	* Reagent A	1.6
2	Air	0.42
3	Serum Sample	0.42
4	* Reagent A	2.0
5	Air	0.6
6	* Reagent B	0.7
7	(To Waste)	2.5

*Reagent A comprised : 2.5 M-methanol in 0.05 M-phosphate, pH 6.9

**Reagent B comprised : 0.82 M-acetylacetone, 2.5 M-methanol in 1.0 M-$NH_4H_2PO_4$, pH 6.0

The nylon-tube co-immobilised enzymes were inserted at position ET, and maintained at 37°C. The 30 turn delay coil was also maintained at 37°C. Extinctions were measured at 412 nm and a sample:wash ratio of 2:1 was used throughout. Unless otherwise specified, samples were assayed at a rate of 60 per hour.

Fig 10b. Flow system used for the determination of serum glucose with separately immobilised nylon-tube derivatives of glucose oxidase and catalase in series.

The composition and flow rates of the various pump-tubing lines was as follows:-

LINE	REAGENT	FLOW RATE (ml. min^{-1})
1	*Reagent A	1.6
2	Air	0.42
3	Serum sample	0.42
4	*Reagent A	2.0
5	Air	0.6
6	**Reagent B	0.7
7	(To Waste)	2.5

*Reagent A comprised : 2.5 M-methanol in 0.05 M-phosphate, pH 6.9

**Reagent B comprised : 0.82 M-acetylacetone, 2.5 M-methanol in 1.0 M-$NH_4H_2PO_4$, pH 6.0.

The nylon-tube immobilised derivatives of glucose oxidase and catalase were inserted at positions ET_1 and ET_2 respectively. The 30 turn delay coil and the enzyme derivatives were maintained at 37°C. Extinctions were measured at 412 nm and a sample:wash ratio of 2:1 was used throughout. Unless otherwise specified, samples were assayed at a rate of 60 per hour.

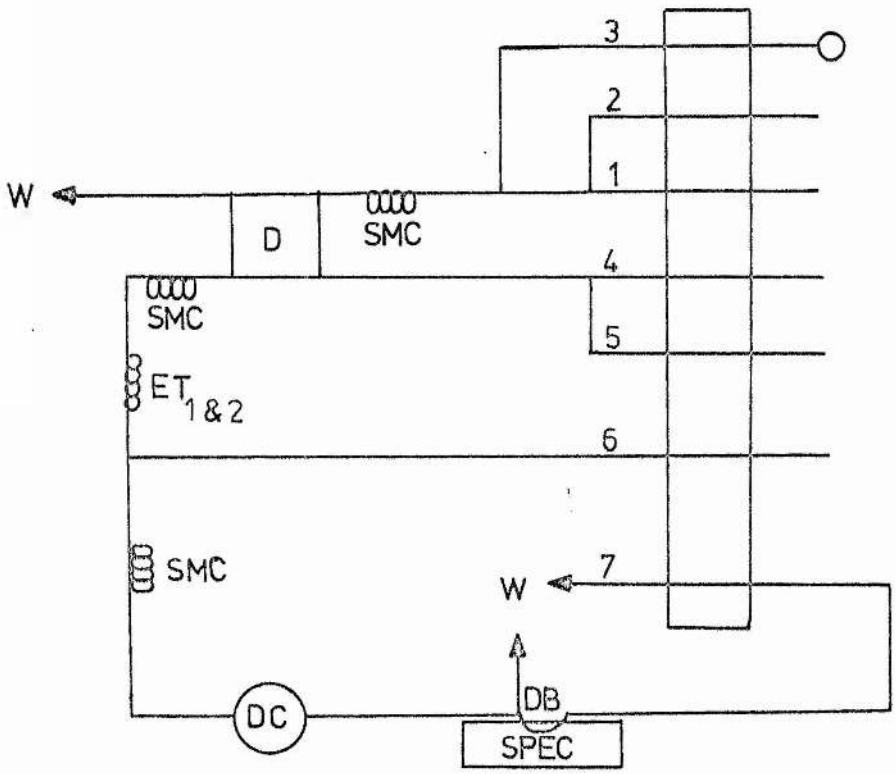


Fig 10(a,b).

Fig 12. Flow system used for the determination of serum glucose using nylon-tube immobilised derivatives of glucose oxida catalase and aldehyde dehydrogenase in series.

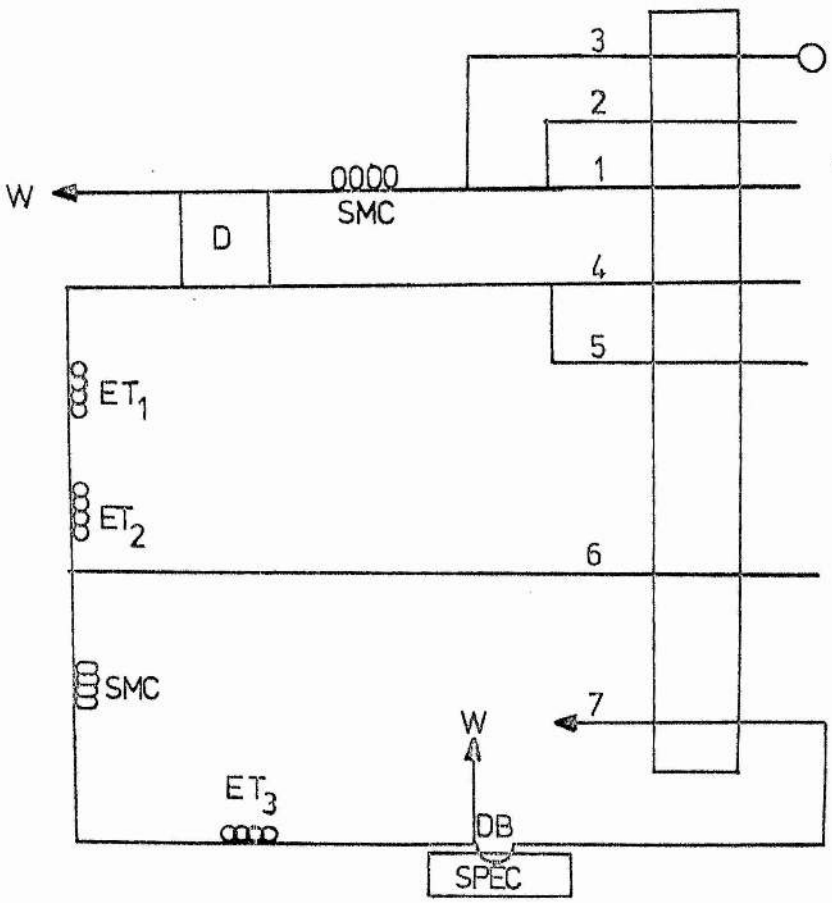
The composition and flow rates of the various pump-tubeing lines were as follows:-

LINE	REAGENT	FLOW RATE (ml min^{-1})
1	*Reagent C	1.6
2	Air	0.6
3	Serum Sample	0.42
4	*Reagent C	2.0
5	Air	0.6
6	**Reagent D	0.23
7	(To Waste)	2.0

*Reagent C comprised : 2.5 M-ethanol in 0.05 M-phosphate, pH 6.9

**Reagent D comprised : 2.5 mM-NAD^+, 2.5 mM-dithioerythritol, 1.0 50 mM-EDTA, in 0.5 Mtris, pH 8.7.

The nylon-tube immobilised derivatives of glucose oxidase, catalase, aldehyde dehydrogenase were inserted at positions ET_1, ET_2, ET_3 respectively, and maintained at a temperature of 37°C. A2:1 sample ratio was used throughout. Extinctions were measured at 340 nm. S were assayed at rates up to 60 per hour.


3
2
1
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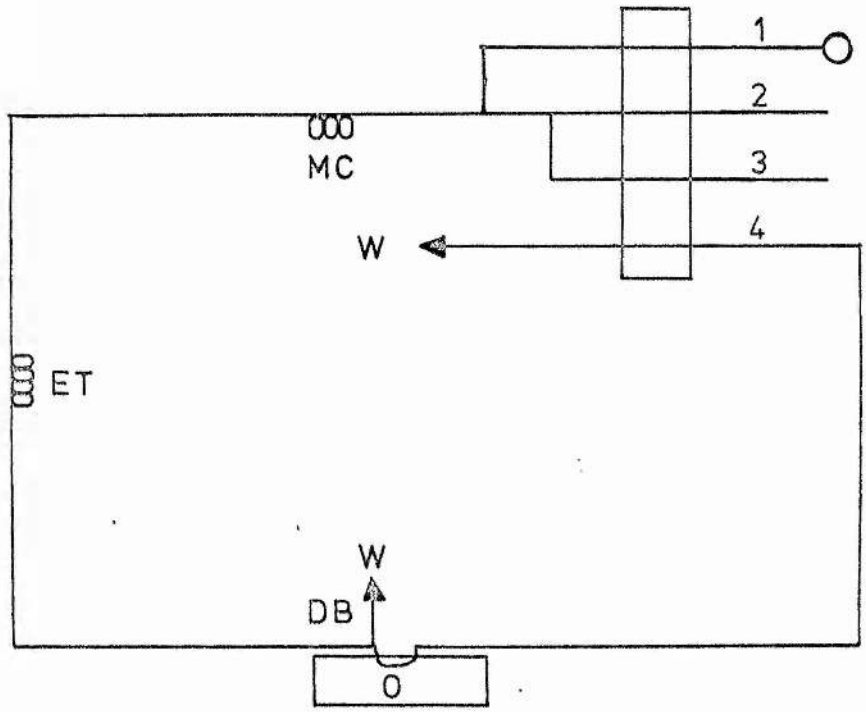
0000
SMC
D
4
5
ET1
ET2
6
SMC
W
7
ET3
DB
SPEC

Fig 13. Flow system used for the determination of glucose using nylon-tube co-immobilised glucose oxidase and catalase in conjunction with a flow-through oxygen electrode.

The composition and flow rate of the various pump-tubing lines were as follows:-

LINE	REAGENT	FLOW RATE (ml min
1	Glucose Sample	0.23
2	0.1 M-Acetate, pH 5.6	2.0
3	Air	0.32
4	(To Waste)	1.6

The nylon-tube co-immobilised glucose oxidase and catalase derivative was inserted at position ET and maintained at 25°C. A sample : wash ratio of 2.1 was used throughout and samples were assayed at rates up to 60 per h.


1
2
MC
3
4
W
ET
W
DB
0

3.7 STABILITY OF NYLON-TUBE IMMOBILISED CATALASE

The stability of nylon-tube immobilised catalase derivatives (DMS alk.) to 1.5 mM-H_2O_2 was determined in the following manner. 3 m lengths of the immobilised enzyme were perfused with 1.5 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9, at a flow rates of 2.9 ml min^{-1} and a temperature of 25°C. The reservoir, containing H_2O_2 was maintained at 5°C by its immersion in a glycol cooling bath. Immediately prior to its passage through the enzyme tube, the temperature was raised to 25°C by means of a water-jacketed delay coil. The tube effluent was collected in 87 ml fractions by means of a fraction collector (Baird and Tatlock Ltd., Chadwell Heath, Essex, England, U.K.). The fractions were stored at 5°C prior to analysis in order to minimise H_2O_2 decomposition. The comcentration of H_2O_2 in the fractions was estimated by means of the acid-KI technique. 0.1 ml aliquots were added to 1.0 ml M-HCl and 1.0 ml 1.0 M-KI. Immediately thereafter the reaction mixture was incubated at 25°C for 15 min. The extinction of the solution was then measured at 349 nm. Standard curves were compiled by subjecting 0.1 ml aliquots of standard H_2O_2 solutions to the above procedure. The stability of nylon-tube immobilised catalase derivatives was expressed in the method described in section 3.5.2.

CHAPTER 4

PREPARATION AND PROPERTIES OF NYLON-TUBE IMMOBILISED CATALASE

The object of the work presented in Chapter 4 is to prepare the most suitable nylon-tube immobilised catalase derivative for use in an autoanalytical system capable of determining hydrogen peroxide concentrations. The sensitivity of such a system depends upon the activity of the immobilised catalase. However other criteria such as the amount of bound protein, the stability of the derivative and the ratio of catalatic : peroxidatic activities displayed by the catalase tube must also be taken into consideration. Consequently the effect of using different alkylation reagents, spacers, bi-functional reagents and sources of catalase upon the bound protein content, catalatic activity, stability and the peroxidatic activity of nylon-tube immobilised catalase derivatives is examined.

4.1. PREPARATION OF NYLON-TUBE IMMOBILISED CATALASE

4.1.1. Adsorption Controls and Enzyme Immobilisation

Ideally, all the protein in a covalently bound immobilised enzyme derivative should be chemically attached to the support. However, adsorption onto the support material can and does occur. Protein attached in this manner can be leached off the support by reagents, during use, thus giving a false impression of the activity and stability of the derivative.

To account for this effect, the following control experiments were performed. 1.4 mg ml^{-1} solutions of catalase were incubated for 3 h at 25°C in 3 m lengths of 1) untreated, 2) ethanolamine-substituted, 3) DMS-alkylated, and 4) glutaraldehyde-activated HMDA-substituted nylon-tubes. All the tubes were submitted to the washing procedure described in section 3.2.4.

The amount of catalase attached and the activity of the derivatives

DERIVATIVE	N/Cat	Nd/E/G/Cat	Nd/HMDA/G/Cat	Nd/Cat
Protein bound (mg)	0	0.2	1.1	2.2
% Total protein bound	0	8	37	73
Protein bound per m ($mg\ m^{-1}$)	0	0.07	0.37	0.71
$K_{app} \times 10^{-3}$ ($cm^2\ min^{-1}$)	0	0	16	0
Specific activity of Immobilised catalase ($cm^2\ min^{-1}\ mg^{-1} \times 10^{-3}$)	0	0	44	0

Table 1. Adsorption and immobilisation of catalase upon nylon-tube. 3 m lengths of nylon-tube immobilised and nylon-tube adsorbed- catalase derivatives were prepared as described in text. In all cases the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. The derivatives were assayed for bound protein and catalatic activity as described in text. The following abbreviations are used in the description of the derivatives : N = untreated nylon-tube ; Nd = DMS-alkylated nylon-tube ; E = ethanolamine ; HMDA = 1,6-diaminohexane ; G = glutaraldehyde ; Cat = catalase.

thus formed were measured by the methods described in sections 3.3 and 3.5.4. The results of these experiments are summarised in Table 1.

The untreated and ethanolamine-substituted nylon-tubes adsorbed 0% and 8% of the total protein respectively, whereas the DMS-alkylated and glutaraldehyde-activated HMDA-substituted nylon-tube derivatives immobilised 73% and 37% respectively. Only the HMDA-substituted derivative retained any enzymic activity, having a K_{app} for catalatic activity of 16×10^{-3} cm^2 min^{-1} corresponding to a protein specific activity of 44×10^{-3} cm^2 min^{-1} mg^{-1}.

The observation that no catalase was adsorbed onto the untreated nylon-tube must be treated with caution. This control takes no account of the chemical reactions to which the nylon-tubes are submitted in the preparation of immobilised enzymes. This treatment of the nylon includes the generation of positive charges and the introduction of hydrophobic spacer compounds, both of which have been shown to enhance the adsorption of protein (15). A stricter comparison with the adsorptive properties of treated nylon-tube can be obtained with the use of monoamine-substituted nylon-tube. Ethanolamine can be attached to nylon through its amine function in the normal manner. However, the absence of free amine functions on the substituted derivative renders the tube inert to any subsequent reactivation procedure. The 8% catalase shown to bind to such a support can only be attached by physical adsorption. From this result the amount of catalase bound by adsorption to the unsubstituted and HMDA-substituted nylon-tubes can be calculated as 9% and 18% respectively, of the total protein. Such values, although high, are unimportant in the case of catalase as no extra activity is imparted to the derivative. For this reason the adsorption of catalase onto nylon-tube immobilised catalase derivatives can be neglected.

Although the glutaraldehyde-activated HMDA-substituted nylon-tube bound only 50% as much catalase as the DMS-activated derivative, only the former retained activity. Campbell (37) and Onyzeli (38) have observed similar effects for several enzymes, where direct immobilisation of the enzyme onto nylon-tube results in little or no activity. This phenomenon can be explained in terms of the proximity of the enzyme to the support, as displacement of the catalase from the nylon surface (e.g. by HMDA-substitution of the nylon) leads to retention of activity. This would explain the inactivity of adsorbed catalase as the positive charges created by the amidine links are most probably one of the prime causes of enzymic adsorption.

Reasons for the deactivation effect can be found by consideration of some properties of the support material. For example, nylon 6 is a hydrophobic polymer and as such could create an unfavorable micro-environment for the catalase. However spacers such as 1,6-diaminohexane are also hydrophobic, and HMDA-substituted catalase tubes have been shown to retain activity. Thus hydrophobicity does not necessarily explain this total deactivation of catalase. Another characteristic of the support material is its bulk and rigidity. This could affect the activity of an immobilised enzyme derivative by the imposition of steric hindrances on the protein. Lilley et al. (39) have shown that the activity of immobilised ficin decreases with larger substrate molecules, suggesting the effect of steric hindrance by the support material. In the case of DMS-activated nylon-tube immobilised catalase, the enzyme is immobilised directly onto the surface of the support, and therefore the possibility of such deactivation is high. Direct attachment of enzyme to supports is not always unfavorable with regards to retention of activity, as one of the most common methods is the coupling to cyanogen bromide-activated sepharose (1). Consideration

Derivative	Nd/HMDA/G/Cat	Nd/DAE/G/Cat	Nd/EAD/G/Cat	Nd/EA/G/Cat	Nd/EA/A/Cat	Nd/Lys/G/Cat	Nd/Lys/G/Cat	Nt/HMDA/G/
Protein bound (mg)	1.1	1.1	1.0	0.8	1.0	1.3	1.2	3.0
% protein bound	37	33	33	27	33	43	40	100
Protein bound per meter tube ($mg\ m^{-1}$)	0.37	0.33	0.33	0.27	0.33	0.43	0.40	1.0
$K_{app} \times 10^{-3}$ ($cm^2\ min^{-1}$)	16	13	13	11	14	19	17	160
Specific activity $\times 10^{-3}$ ($cm^2\ min^{-1}\ mg^{-1}$)	44	40	40	40	44	44	42	54

Table 2. The preparation of nylon-tube immobilised catalase derivatives. 3 m lengths were prepared under identical conditions as described in text. In all cases the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase in 0.2M-phosphate, pH 7.8. Each derivative was assayed for bound protein and catalatic activity as described in text. The following abbreviations are used : Nd = DMS-alkylated nylon-tube ; Nt = TCTFB-alkylated nylon-tube ; HMDA = 1,6-diaminohexane ; DAE = 1,2-diaminoethane ; EA = egg albumen ; EAD = denatured egg albumen ; Lys = lysine ; G = glutaraldehyde ; A = 2,3-pimelate ; Cat = catalase.

of the structures of sepharose and nylon however, provides an explanation for such differences. Sepharose is swollen, flexible and hydrophilic whilst nylon is hydrogen-bonded, tightly packed and rigid. Steric hindrance will therefore be more prevalent in a support made from the latter material.

4.1.2. Effect of Diamine Spacers on the Activity of Catalase Tubes

The results of the previous section indicate that catalase has to be displaced a certain distance from the surface of the nylon before activity is retained. The effect of variation of this distance upon the activity of the derivatives was investigated by use of different spacer compounds. 3 m lengths of DAE- and HMDA-substituted nylon-tube were prepared under identical conditions in the manner described in section 3.2. Both were activated with glutaraldehyde, incubated with catalase and washed as described in section 3.2.4. The amount of bound protein and the catalatic activity of each derivative were assayed as described in sections 3.3 and 3.5.4. These results are summarised in Table 2.

The DAE- and HMDA-substituted nylon-tube immobilised catalase derivatives bound approximately the same amount of protein, but displayed catalatic activities of 13 x 10^{-3} cm^2 min^{-1} and 16 x 10^{-3} cm^2 min^{-1} respectively. This corresponded to protein specific activities of 40 x 10^{-3} cm^2 min^{-1} mg^{-1} and 44 x 10^{-3} cm^2 min^{-1} mg^{-1} respectively. Thus use of HMDA in place of DAE resulted in a 23% increase in tube activity. This increase can be explained by the change in distance of catalase from the support in the two derivatives, the former comprising 6 carbon atoms to the two carbon atoms of the latter. This emphasises the importance of the displacement distance of the catalase and its relationship to retention of activity.

4.1.3. Effect of L-Lysine Spacers on the Catalatic Activity of Catalase Tubes

This amino acid was attached to the support and subsequently activated in a similar manner to that described for diamines in section 3.2. This spacer compound differed from ones used previously owing to the negative charge created by the presence of the α-carboxyl group. A lysine-substituted nylon-tube immobilised catalase derivative was prepared under identical conditions to those previously described for DAE- and HMDA-substituted derivatives. The amount of protein bound and the catalatic activity of the derivative were assayed by the methods described in sections 3.3 and 3.5.4. These results also are shown in Table 2. 43% of the total protein was immobilised to the derivative giving a tube activity of 19×10^{-3} cm^2 min^{-1}, corresponding to a protein specific activity of 44×10^{-3} cm^2 min^{-1} mg^{-1}.

6% more protein was bound to this derivative than with the HMDA-substituted nylon-tube. This imparted a higher total activity but identical protein specific activity. These observations suggest that masking of the positive charges by the incorporation of carboxyl groups into substituted nylon-tube derivatives does not increase the specific activity of protein bound to the support.

Di- lysyl spacers were used to further increase the negative charge and spacer length. A lysine-substituted nylon-tube derivative was activated with glutaraldehyde in the normal manner and incubated with a further solution of lysine. After removal of excess amino acid, the tube was again reactivated with glutaraldehyde and incubated with a 1.4 mg ml^{-1} solution of catalase. Subsequent treatment was identical to that described previously. The derivative was assayed for bound protein and catalatic activity as described in sections 3.3 and 3.5.4.

The results are shown in Table 2. 40% of the total protein was

immobilised to the derivative giving a tube activity of 17 x 10^{-3} cm^2 min^{-1} corresponding to a protein specific activity of 42 x 10^{-3} cm^2 min^{-1} mg^{-1}. Thus the addition of further negative charges to the support decreased the protein specific activity even though the displacement distance of the enzyme from the nylon surface was increased in the process. Another factor that may influence the activity of this derivative is the inclusion of an extra glutaraldehyde-activation procedure. Although, in principle, 100% yields may be expected, in practise they are never attained. The efficiency of the preparation of the tube for attachment of catalase may therefore be impaired.

4.1.4. Effect of Egg Albumen Spacers on Activity of Catalase Tubes

The effect of using protein as a spacer was also studied. Glutaraldehyde activated protein-substituted nylon-tube was incubated with a 1.4 mg ml^{-1} solution of catalase, washed and assayed for bound protein and catalatic activity as described in sections 3.3 and 3.5.4. The results are shown in Table 2. Only 27% of the total protein was immobilised to the support which had a catalatic activity of 11 x 10^{-3} cm^2 min^{-1} corresponding to a protein specific activity of 40 x 10^{-3} cm^2 min^{-1} mg^{-1}. When the protein spacer was denatured immediately prior to glutaraldehyde activation as described in section 3.2, 33% of the total protein was immobilised and the derivative had a catalatic activity of 13 x 10^{-3} cm^2 min^{-1} corresponding to a protein specific activity of 40 x 10^{-3} cm^2 min^{-1} mg^{-1}. Thus, when compared with the results obtained with HMDA-substitution, the tube activities of the denatured and undenatured protein-substituted catalase derivatives decreased by 19% and 31% respectively. As a protein spacer can be considered as a 'poly-amine', the possibility exists in theory that

it could be attached to the support by one of its amine functions and present several more for subsequent activation and attachment of enzyme. In this way a higher spacer:enzyme ratio than 1:1 could be realised. However the decreased levels of enzyme bound to such supports suggest that the protein is being attached to the support through more than one amine function and rendering many of the remainder inaccessable to glutaraldehyde activation. Denaturation of the protein spacer increases the quantity of protein bound. This is probably due to the uncovering of amine functions previously embedded in the molecule. Therefore no advantages accrue from the use of protein spacers with regard to derivative activity.

4.1.5. Comparison of Bifunctional Reagents used in the Preparation of Catalase Tubes

The nylon-tube immobilised catalase derivatives discussed previously were all prepared by the attachment of catalase to the support via glutaraldehyde. Although this method has been most commonly used in the preparation of nylon-tube immobilised enzymes (40-43) other cross-linking reagents are also suitable. For example, Morris and Hornby have demonstrated the use of bisimidates in such a role (44). The two activation methods for the preparation of catalase tubes were studied in the following manner. A 6 m length of protein-substituted nylon-tube was prepared in section 3.2. One 3 m portion was activated with glutaraldehyde as described in section 3.2.3.1, whilst the other was activated with diethyladipimidate as described in section 3.2.3.2. In each case the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. After washing, the amount of protein bound and the catalatic activity of the derivatives were assayed as described in sections 3.3 and 3.5.4. The results are summarised in Table 2. The diethyladipimidate and

glutaraldehyde-activated nylon-tube immobilised catalase derivatives bound 35% and 27% of the total protein respectively corresponding to protein specific activities of 44 x 10^{-3} cm^2 min^{-1} mg^{-1} and 40 x 10^{-3} cm^2 min^{-1} mg^{-1} respectively.

A 6% increase in bound protein was realised by the bisimidate activated tube. This suggests that diethyladipimidate is more effective at binding protein than glutaraldehyde. The reaction mechanism of the latter reagent is still incompletely understood, various side-reactions being known to occur (45). This may account for the less efficient utilisation of amine functions.

The use of bisimidates for the activation of substituted nylon-tube also resulted in a 10% increase in protein specific activity. This may be due to the difference in linkage of the enzyme to the substituted nylon-tube. It is possible that the glutaraldehyde reaction may involve not only free amine functions but also with other amino acid residues in proteins. Consequently, enzymes immobilised in this way may be bound to the support material in a more random manner, thus increasing the possibility of involvement of amino acids essential for catalytic activity.

In practise, however, it has been found more convenient to activate substituted nylon-tubes with glutaraldehyde owing to the simplicity of the process and storage of the reagents. Glutaraldehyde is commercially available and stable at 4°C indefinitely, whereas diethyladipimidate requires synthesis, is labile, and has to be stored under strict dessication to prevent hydrolysis. This susceptibility to moisture also requires that the substituted nylon-tube be thoroughly dried prior to its activation, thus neccessitating another stage in the preparation procedure. For simplicity of description, unless otherwise mentioned, all immobilised enzyme derivatives subsequently mentioned in

this thesis were prepared by glutaraldehyde-activation.

4.1.6. O-Alkylation of Nylon-Tube Using TOTFB and DMS

The relative merits of DMS and TOTFB as alkylating reagents in the preparation of catalase tubes were studied in the following manner. One 3 m length of HMDA-substituted nylon-tube was prepared by DMS-alkylation, and another by TOTFB-alkylation as described in section 3.2. Subsequent activation, enzyme coupling and washing stages were as described in section 3.2.4, the enzyme coupling solution comprising 1.4 mg ml^{-1} catalase in each case. The amount of protein bound and the catalatic activities of the derivatives were assayed as described in sections 3.3 and 3.5.4. These results are summarised in Table 2. The TOTFB- and DMS- alkylated nylon-tubes bound 100% and 30% respectively of the total catalase, with tube activities of 160 x 10^{-3} cm^2 min^{-1} and 16 x 10^{-3} cm^2 min^{-1} respectively. These values gave protein specific activities of 160 cm^2 min^{-1} mg^{-1} and 44 x 10^{-3} cm^2 min^{-1} mg^{-1}. These results clearly indicate that the o-alkylation of nylon-tube with TOTFB produces a derivative with a 60% increase in bound enzyme, a 300% increase in specific activity, and a total tube activity increase of 1000% when compared with the corresponding derivative prepared by DMS-alkylation.

TOTFB has previously been found to be far more efficient than DMS in the preparation of several other nylon-tube immobilised enzymes (46,47). The marked differences produced by the two different alkylation methods can be explained in terms of the relative strength of the two reagents. DMS-alkylation of nylon has to be performed at a temperature of 100°C, negligable modification of the nylon being attained at lower temperatures (48). TOTFB can be used at room-temperature with a longer reaction time that allows the alkylation

reaction to be more accurately controlled. The temperature of the alkylation reaction is important as secondary imidates are extremely sensitive to hydrolysis, this sensitivity increasing with temperature as does the water permeability of nylon (49). In the DMS-alkylation procedure, the 100°C reaction temperature required attained by the immersion of the nylon-tube in a boiling water bath. Thus it is a possibility that a large fraction of the secondary imidates formed are immediately hydrolysed.

TOTFB-alkylation of nylon obviates these problems and the concentration of secondary imidates formed is probably much higher. This will provide more active sites on the surface of the nylon for spacer attachment and consequently more catalase will be immobilised to the support. Such an increase in bound enzyme may also beneficially affect the micro-environment of the immobilised enzyme by more efficient shielding of any support surface effects on the enzyme. The 25% increase in protein specific activity obtained by TOTFB alkylation may be the result of such effects.

TOTFB is also a far safer reagent to handle. Although it must be considered as poisonous, unlike DMS it does not cause burns or give off poisonous vapours.

For reasons of simplicity in the description of nylon-tube immobilised enzymes, subsequent derivatives mentioned in this thesis were prepared by TOTFB alkylation unless otherwise stated.

4.1.7. Effect of Bound Protein Concentration on the Catalatic Activity of Immobilised Catalase

The results of the previous section showed that up to 3 mg of catalase could be bound to a HMDA-substituted nylon-tube. The following experiment was undertaken in order to determine the effect of immobilising different quantities of catalase on both the protein

Coupling solution ($mg\ ml^{-1}$)	0.03	0.1	0.18	0.70	1.04	1.5
Protein bound (mg)	0.06	0.21	0.39	1.20	2.22	3.2
Protein bound per meter tube ($mg\ m^{-1}$)	0.02	0.07	0.13	0.40	0.73	1.0
$K_{app} \times 10^3$ ($cm^2\ min^{-1}$)	18.0	40.0	50.0	52.0	52.3	53.0
Protein specific activity $\times 10^3$ ($cm^2\ min^{-1}\ mg^{-1}$)	900	570	380	130	72	40

Table 3. Variation of catalase concentration in coupling solution
3 m lengths of HMDA-substituted nylon-tube immobilised catalase
derivatives were prepared under identical conditions as described
text. The concentration of catalase in the coupling solution was
varied from 0.03 mg ml^{-1} to 1.5 mg ml^{-1} and the resulting derivati
assayed for bound protein and catalatic activity as described in

specific activity and overall catalatic activity of the immobilised catalase. Six 1 m lengths of HMDA-substituted nylon-tube immobilised catalase were prepared as described in section 3.2. The enzyme coupling solutions comprised 0.03, 0.1, 0.18, 0.7, 1.04 and 1.5 mg ml^{-1} of protein respectively. The amount of bound protein and the catalatic activity of each derivative were measured as described in sections 3.3 and 3.5.4. The results are shown in Table 3. In all cases 100% of the total protein was immobilised to the support. Initially, increasing the bound catalase from 0.02 mg m^{-1} to 0.13 mg m^{-1} resulted in a rapid increase in the overall tube activity. However above 0.13 mg m^{-1} bound catalase the overall activity increased by only 6% for an eight-fold increase in immobilised catalase. As the protein bound per meter of tube was increased from 0.02 - 1.07 mg m^{-1}, the protein specific activity dropped from 900 cm^2 min^{-1} mg to 40 cm^2 min^1 mg^{-1}.

Two effects are therefore apparent : 1) initially the overall activity of the catalase tubes rapidly increased with bound protein but then plateaued, 2) the specific activity of the immobilised catalase dropped with increasing amounts of bound protein. These two observations can be explained by the existence of several types of enzyme binding sites on the nylon-tube. It is most likely that the inside surface of such a tube is not a flat smooth plane. At the micro level it will be a rough surface containing many fissures, the occurrence of which will probably be accentuated by the alkylation process. For reasons of simplicity, enzymes are thought of as being immobilised upon the surface of the tube. In practise, they are probably bound at various levels in the nylon, with the majority of activity residing in the molecules attached to the more exposed regions of the nylon surface. In the case of catalase, it has been demonstrated

that molecules in close proximity to the support will not retain activity. Thus the surface sites would not only be the first to immobilise enzyme through the spacer, but also contain the majority of the immobilised activity. Small amounts of immobilised protein would therefore display the same activity as larger amounts, once the surface sites were filled, and this would result in a decrease in protein specific activity of the immobilised catalase. Another two factors may also contribute to the absence of extra activity on increasing the quantity of immobilised protein. Overcrowding of enzyme molecules on the surface of the nylon may prevent necessary orientations from occurring due to the close proximity of enzyme molecules to each other. Secondly, diffusional effects may take place with enzyme molecules embedded in the nylon. In this case, the rate of reaction would depend upon the diffusion of H_2O_2 to the enzyme, and not upon the catalatic activity of the immobilised catalase.

4.1.8. Effect of Tube Diameter on the Catalatic Activity of Catalase Tubes

One consequence of the surface site hypothesis suggested in the previous section is that an increase in surface area of the tube should result in an increase in catalatic activity. This effect was studied with the use of 1 mm and 2 mm bore nylon-tube. A 1 mm length of each tube was substituted with HMDA and enzyme was attached in the manner described in section 3.2. The coupling solution of the 1 mm and 2 mm bore derivatives comprised 1.4 mg ml^{-1} and 0.34 mg ml^{-1} catalase respectively. In this manner 1.0 mg catalase per meter of each derivative was available for immobilisation. After washing, the amount of bound protein and catalatic activities were assayed as described in sections 3.3 and 3.5.4.

The 1 mm and 2 mm bore nylon-tube immobilised catalase derivatives

Derivative	Nt/H/G/Cat	Nt/S/G/Cat	Nt/A/G/Cat	Nt/A/Cat	Nt/A/AZ/Cat	Nt/HMDA/G/Cat
Protein bound (mg)	1.9	1.4	1.4	0	0.2	3.0
% protein immobilised	63	47	47	0	7	100
Protein bound per meter tube ($mg\ m^{-1}$)	0.63	0.47	0.47	0	0.07	1.0
$K_{app} \times 10^{-3}$ ($cm^2\ min^{-1}$)	50	100	160	0	120	160
Specific activity $\times 10^{-3}$ ($cm^2\ min^{-1}\ mg^{-1}$)	79	210	333	0	1714	54

Table 4. Effect of dihydrazide spacers on the activity of nylon-tube immobilised catalase. 3 m lengths were prepared under identical conditions as described in text. In all cases the enzyme coupling solution comprised 1.4 mg ml^{-1} in 0.2 M-phosphate, pH 7.8. Each derivative was assayed for bound protein and catalatic activity as described in text. The following abbreviations are used in the description of the derivatives : Nt = TOTFB-alkylated nylon-tube ; H = hydrazine ; S = succinic acid dihydrazide ; A = adipic acid dihydrazide ;

each bound 100% of the total protein with catalatic activities of 18×10^{-3} cm^2 min^{-1} and 30×10^{-3} cm^2 min^{-1} respectively, corresponding to protein specific activities of 18×10^{-3} cm^2 min^{-1} mg^{-1} and 30×10^{-3} cm^2 min^{-1} mg^{-1} respectively. Thus a two-fold increase in surface area resulted in a 67% increase in tube activity. This suggests that the surface site theory may be operative in nylon-tube immobilised derivatives.

The increased internal diameter of the tube also results in an increase in volume. This could affect the apparent enzymic activity, by extending the dwell-time of substrate per unit length of tube. However, measurement of derivative activity by the recirculation assay described in section 3.5.1 obviates this problem.

4.1.9. Effect of Dihydrazide Spacers on the Catalatic Activity of Catalase Tubes

The effect of dihydrazides and hydrazine as spacer compounds on the activity of nylon-tube immobilised catalase was studied in the following manner. 3 m lengths of 1) hydrazine-, 2) succinic acid dihydrazide-, 3) adipic acid dihydrazide-substituted nylon-tube immobilised catalase derivatives were prepared as described in section 3.2. In each case the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. The results are summarised in Table 4. The hydrazine-, succinic acid dihydrazide- and adipic acid dihydrazide-substituted nylon-tubes bound 63%, 47% and 47% of the total protein respectively. The tubes had catalatic activities of 50×10^{-3} cm^2 min^{-1}, 100×10^{-3} cm^2 min^{-1}, and 160×10^{-3} cm^2 min^{-1} respectively, corresponding to protein specific activities of 79×10^{-3} cm^2 min^{-1} mg^{-1}, 208×10^{-3} cm^2 min^{-1} mg^{-1}, and 333×10^{-3} cm^2 min^{-1} mg^{-1}. Therefore increasing catalase displacement from the nylon surface increased the activity retained by the immobilised enzyme. This result

alkylated nylon-tube H = hydrazine ; S = succinic acid dihydrazide ; A = adipic acid dihydrazide

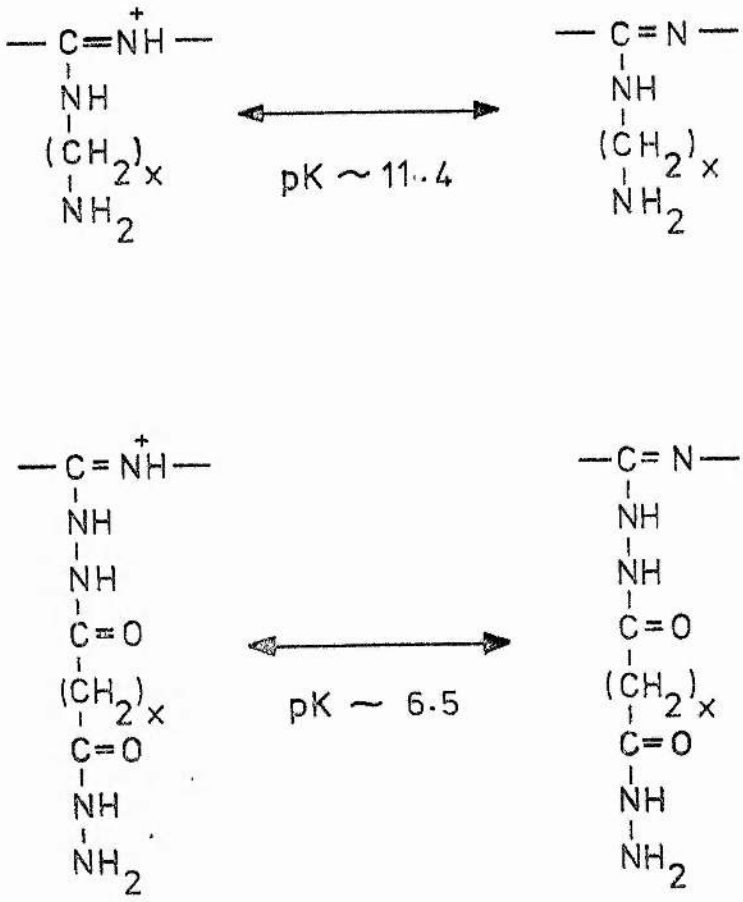


Fig 14. Acid-base properties of amidines and amidrazones.

is consistant with that reported previously in section 4.1.2 for diamine spaced nylon-tube immobilised catalase derivatives.

53% less protein was bound to the adipic dihydrazide substituted nylon tube than the HMDA-substituted tube. The reasons for this difference may be due to the relative insolubility of dihydrazides in organic solvents. Whereas for diamine substitution of alkylated nylon a solution comprising 1.0 M-HMDA is used, the maximum solubility of adipic dihydrazide attained was 15 mM in formamide. It is probable that the smaller concentration of the latter could affect the yield of substituted nylon-tube. This in turn would affect the quantity of enzyme subsequently bound to such a support.

Although the hydrazide-substituted catalase tubes contained less bound protein, than HMDA-substituted derivatives their catalatic activity was of the same order due to a two-fold increase in their protein specific activity.

The extent of adsorption of protein on dihydrazide-substituted nylon-tube was investigated in the following manner. A 3 m length of adipic acid dihydrazide substituted nylon-tube was prepared as described in section 3.2. The reactivation procedure was omitted and a 1.4 mg ml^{-1} catalase solution incubated in the tube. After washing, the derivative was assayed for bound protein and catalatic activity as described in sections 3.3 and 3.5.4. The results, summarised in Table 4, show the absence of any enzymic adsorption onto the support. Comparison of Tables 1 and 4 shows that diamine- and dihydrazide-substituted derivatives have markedly different adsorptive properties. The reasons for these contrasting adsorptive characteristics may lie in the nature of the amidine and amidrazone bonds shown schematically in Fig 14. The pK of the amidine link is greater than 11.4 (50.) whilst

that of the amidrazone is around pH 6.5 (51). Thus at neutral pH and above, there will be little or no positive charge on the amidrazone bond owing to delocalisation of the electrons, whilst the amidine function retains its positive charge throughout the pH range at which enzymes are both immobilised and used. This suggests that it is the positive charge of the substituted nylon-tubes that causes adsorption of the enzyme.

The use of dihydrazide-substituted nylon-tubes also permits the direct attachment of catalase to the substituted nylon-tube without recourse to cross-linking reagents. A 3 m length of azide-activated succinic acid dihydrazide-substituted nylon-tube was prepared as described in section 3.2. The enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. After washing, the derivative was assayed for bound protein and catalatic activity as described in sections 3.3 and 3.5.4. The results are summarised in Table 4. 7% of the total enzyme was bound and the immobilised derivative had a catalatic activity of 12×10^{-3} cm^2 min^{-1}, corresponding to a protein specific activity of 171×10^{-2} cm^2 min^{-1} mg^{-1}.

Thus the azide activation bound 40% less protein than the corresponding glutaraldehyde activated derivative, but the catalatic activity fell by only 20%, due to a 500% increase in the protein specific activity. Whilst this increase is probably due to the decreased quantity of bound protein, it may also reflect the effect of the different immobilisation process, on the retention of activity.

Although azide activation serves to underline the versatility of dihydrazide-substituted derivatives, the utility of such a catalase derivative is limited owing to the small quantities of enzyme bound to the support.

SUMMARY OF SECTION 4.1

The alkylation of the nylon was performed by two different reagents, TOTFB and DMS. Use of the former was found to increase the catalatic activity of catalase tubes by an order of magnitude over corresponding DMS-alkylated derivatives.

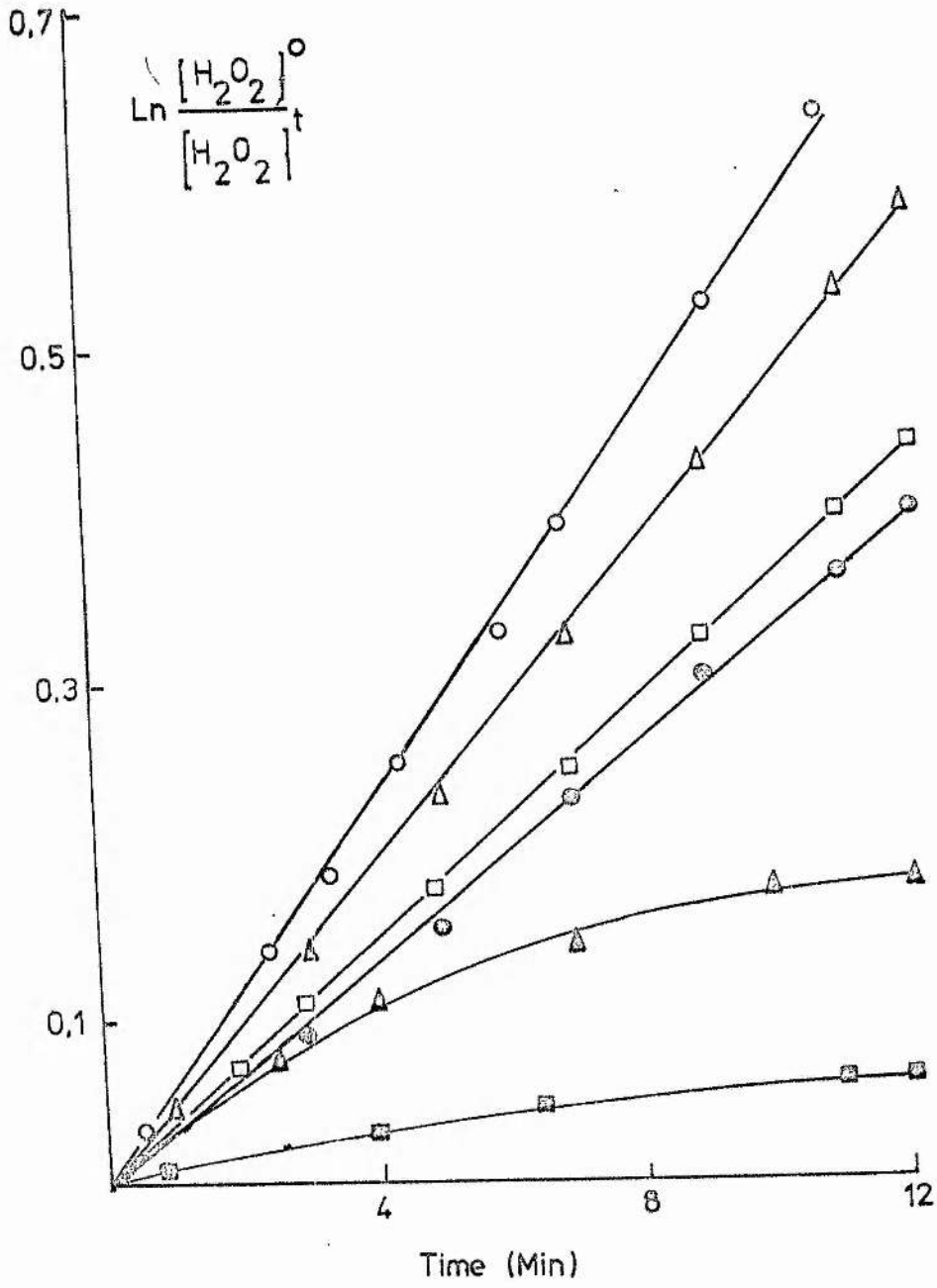
Although direct attachment of catalase to the nylon-tube resulted in inactivation of the enzyme, the substitution of nylon-tube with spacers enabled an active immobilised enzyme to be prepared by attachment of the catalase to the substituted nylon via a bi-functional reagent. The effect of various spacer compounds on the catalatic activity of immobilised catalase was examined, and it was found that an increase in the length of spacer employed resulted in greater retention of enzyme activity. In general HMDA-substitution of the TOTFB-alkylated nylon-tube was found to produce a derivative with high enzymic activity and bound protein content.

The use of different bifunctional reagents for the attachment of enzyme to the substituted nylon tube was also examined. Although diethyladipimidate immobilised 6% more protein to the support than glutaraldehyde, the latter reagent was found to afford a more convenient method due to its ease of storage and stability.

The effect of tube diameter on the catalatic activity of immobilised catalase was studied with the use of 1 mm and 2 mm-base nylon tubes. The latter, containing the greater surface area, exhibited 60% more activity than the former.

Consideration of the data obtained in this section, shows that the most catalatically active derivative prepared was glutaraldehyde activated/HMDA-substituted/2 mm-base nylon tube immobilised catalase.

Fig 15 . Stability of lysine-substituted nylon-tube immobilised catalase (DMS-alk.) to intermittent assay in the presence of 1.5 mM-H_2O_2. ○ Activity immediately after preparation ; △ after 1 h ; □ after 2.5 h ; ◉ 4.5 h ; ▲ 24 h ; ■ 30 h .


0,7
0.5
0.3
0,1
$\text{Ln} \frac{[H_2O_2]^o}{[H_2O_2]^t}$
4
8
12
Time (Min)

Time of Assay After Preparation (h)	Percentage K_{app} Remaining				
	Lys	DAE	HMDA	$(Lys\ Glut)_2$	EA
0	100	100	100	100	100
1.5	84.2	83.2	–	82	76
2.5	63.2	68.7	–	–	–
3.5	56.1	–	86.7	66	–
17	–	41.7	–	40	–
21	– Non Linear		56.1	–	71.8
24	Non Linear	–	–	29	70.5
30	Non Linear	–	40.0	–	–
42	–	–	–	–	70

Table 5. Stability of nylon-tube immobilised catalase to intermi assay and storage. Derivatives were prepared by DMS alkylation an glutaraldehyde activation and assayed under identical conditions a described in text. In all cases the enzyme coupling solution comp 1.4 mg ml^{-1} catalase in 0.2 M-phosphate, pH 7.8. The following abbreviations are used : Lys = Lysine-spaced ; DAE = 1,2-diamino spaced ; HMDA = 1,6-diaminohexane spaced ; $(Lys\ Glut)_2$ = Dilysino spaced ; EA = protein spaced catalase tube.

4.2. STABILITY OF NYLON-TUBE IMMOBILISED CATALASE PREPARED BY DMS-ALKYLATION

4.2.1. Stability of Immobilised Catalase (DMS Alk.) to Intermittant Assay in the Presence of 1.5 mM-H_2O_2

The stability of nylon-tube immobilised catalase to intermittant assay and storage was studied in the following manner. 3 m lengths of HMDA-, DAE-, Lys-, DiLys-, and protein-substituted catalase tubes were prepared as described in section 3.2. In each case the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. Immediately after washing, each derivative was assayed by the recirculation assay described in section 3.5.4, perfused with 0.05 M-phosphate, pH 6.9, and stored filled with the same reagent at 9°C. At various times after their preparation, each derivative was re-assayed and stored under identical conditions. The stability of the derivatives was monitored by measurement of the K_{app} for catalase activity as described in section 3.5.1. A typical set of plots is shown in Fig 15. The decreasing slopes of the lines represent the decreasing K_{app} and therefore the decreasing activity of the immobilised catalase derivative. The results obtained for all the derivatives tested are summarised in Table 5. The DAE-, HMDA-, and Lys-substituted catalase tubes lost the majority of their activity over 30 h (5 assays) whilst the protein-substituted tube retained 70% of its activity over a similar period of time. The former tubes exhibited non-linear responses prior to complete deactivation. This may be due to the loss of enzymic activity during the assay itself. It is likely that this effect will be most pronounced when only small quantities of active enzyme remain.

4.2.2. H_2O_2 Deactivation of Nylon-Tube Immobilised Catalase

The possible causes of the lack of stability associated with these

immobilised catalase can be separated into two classes. An inherent instability may be caused by the immobilisation process itself. For example, only one of a number of sub-units may be immobilised, causing the enzyme to lose its structural integrity by sub-units being leached off the support by reagents during use of the immobilised enzyme.

The second possibility is the occurrence of a substrate deactivation where exposure to H_2O_2 is the cause of loss in activity. The latter is known to occur in the soluble enzyme system (52).

In order to determine whether some support effect was the cause of instability, the following experiment was undertaken. Duplicate 3 m lengths of HMDA-substituted catalase tubes (DMS-alk) were prepared as described in section 3.2. One was submitted to the procedure described in the previous section whilst the other was continually perfused with 0.05 M-phosphate, pH 6.9, over a similar period of time. The former derivative was inactivated over a 50 h period whereupon the latter was assayed for the first time. The activity of the tube perfused with 0.05 M-phosphate, pH 6.9 corresponded to the initial activity of the deactivated derivative. This result suggests the absence of any adverse support effect on the stability of immobilised catalase.

The possibility of a substrate effect was then studied in the following manner. A 4 m length of lysine-substituted catalase tube (DMS alk) was prepared as described in section 3.2. The enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. The derivative was divised into four 1 m sections which were assayed individually by the single pass of 1.5 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9 at a flow rate of 1.5 ml min^{-1}. The segments were placed in series and perfused with 1.5 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9 as described in section 3.7. At time intervals, the derivative was washed with 0.05 M-phosphate, pH 6.9 and the individual segments re-assayed as described above. They were then reassembled in the same order and

their perfusion with 1.5 mM-H_2O_2 continued. The decrease in activity of each of the segments over a 10.5 h of exposure to H_2O_2 is summarised in Table 6.

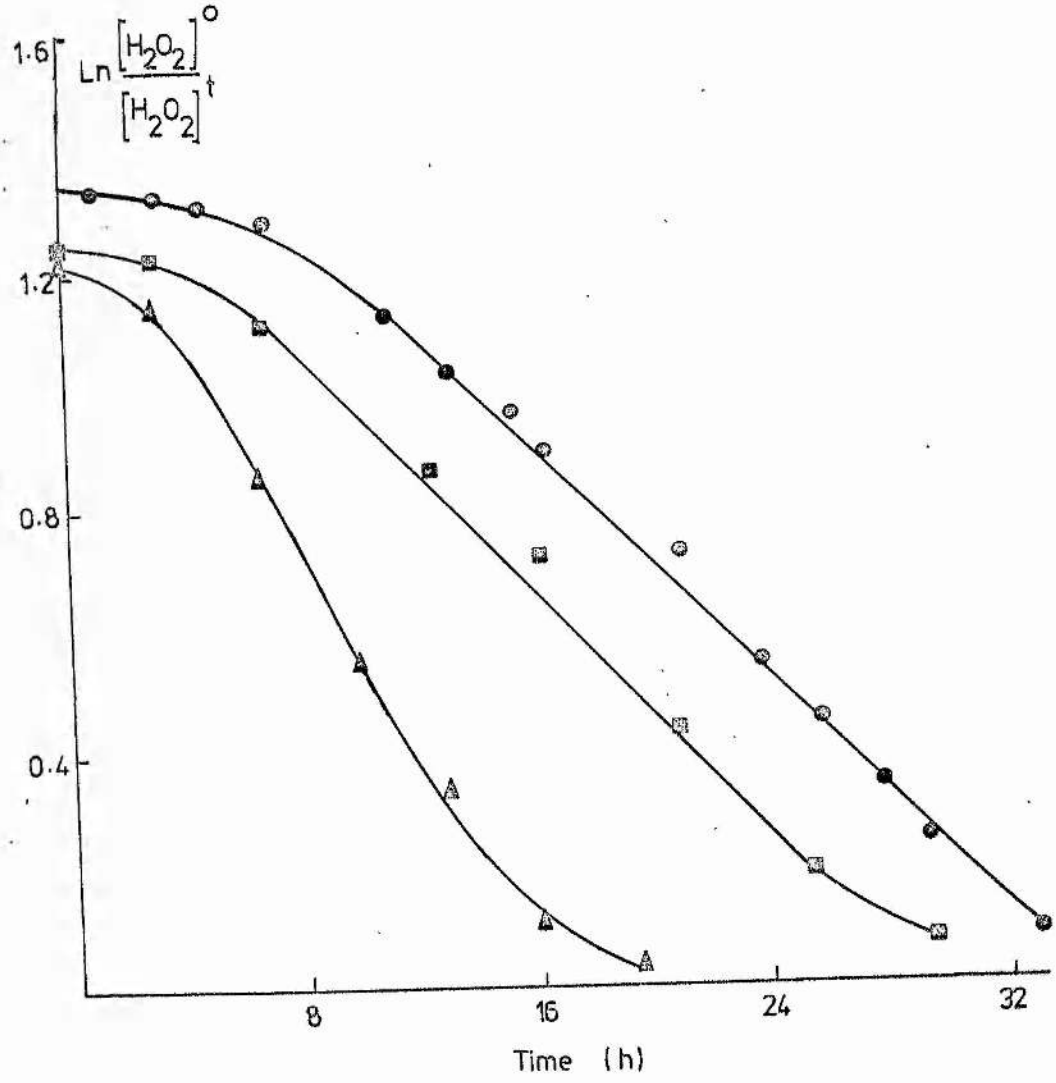
	Percentage K_{app} Remaining at time			
Time (h)	0	4.0	7.0	10.5
Segment 1	100	43	6	6
Segment 2	100	56	44	21
Segment 3	100	60	47	34
Segment 4	100	67	54	40

Table 6. Deactivation of nylon-tube immobilised catalase by H_2O_2. A lysine-substituted nylon-tube immobilised catalase derivative was cut into four 1 m sections which were assayed individually, rejoined in order, and perfused with 1.5 mM-H_2O_2 at pH 6.9 as described in text.

The leading segment (number 1) retained 43%, 6%, and 6% of its activity over 4 h, 7 h, and 10.5 h respectively. The rear segment (number 4) retained 67%, 54%, and 40% of its activity over identical periods. Thus segment 1 was deactivated at a faster rate than segment 4. This observation is in keeping with a substrate deactivation effect where the concentration of substrate is related to the rate of inactivation.

An H_2O_2 concentration gradient exists across the nylon-tube immobilised catalase derivative due to substrate utilisation. Thus the rate of any deactivation effect dependant upon substrate concentration must also vary with this gradient. The pattern of deactivation observed therefore shows the presence of H_2O_2 deactivation of the immobilised catalase derivative.

Fig 16 . Effect of spacers upon the stability of nylon-tube immobilised catalase derivatives (DMS-alk.) to continuous perfusion with 1.5 mM-H_2O_2 at pH 6.9 . ▲ HMDA-substituted ; ▣ Egg albumen-substituted ; ◉ Denatured egg albumen-substituted catalase tube.


1.6
Ln [H2O2]° / [H2O2]t
1.2
0.8
0.4
8
16
24
32
Time (h)

4.2.3. Stability of Nylon-Tube Immobilised Catalase (DMS alk) to Continuous Perfusion with 1.5 mM-H_2O_2.

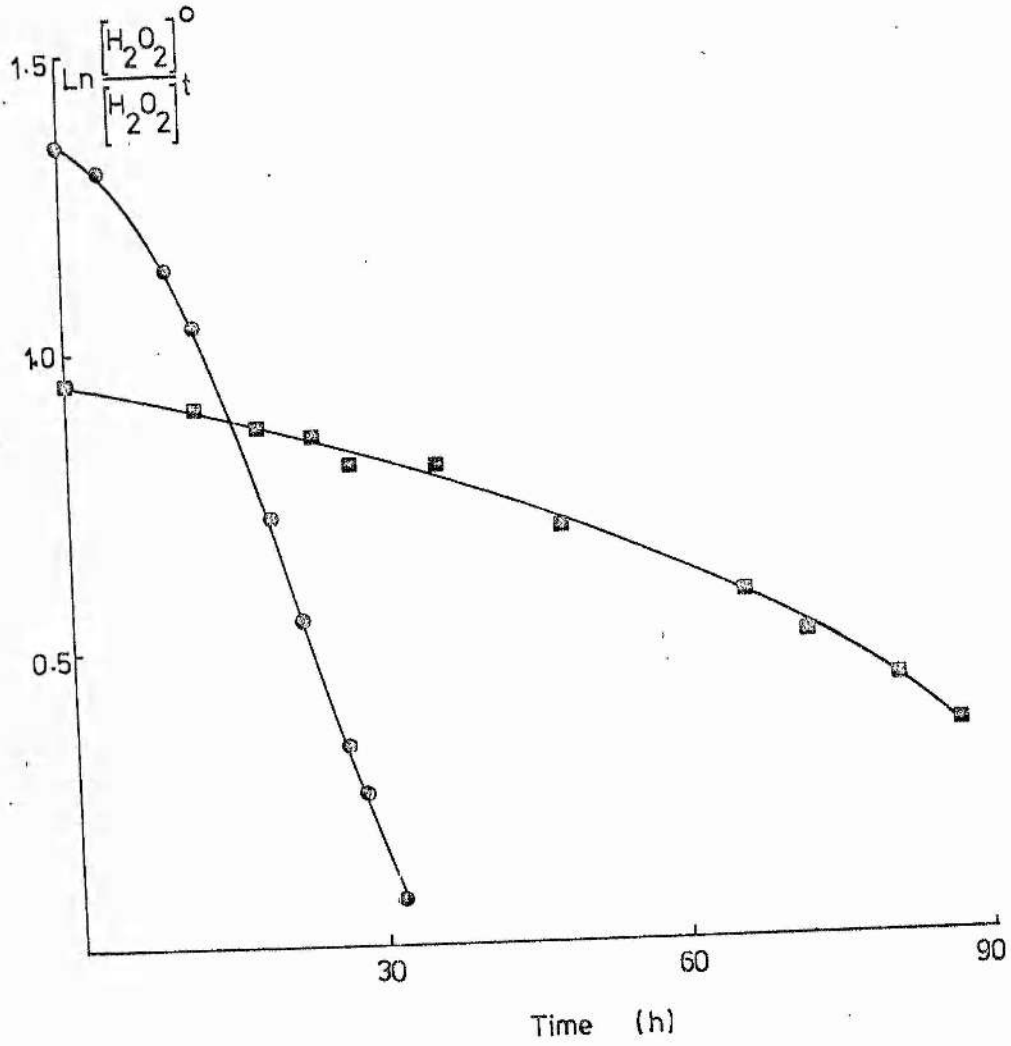
The stability of nylon-tube immobilised catalase derivatives to continuous exposure with 1.5 mM-H_2O_2 at pH 6.9 was studied by the method described in section 3.7. HMDA-, protein-, and denatured protein-substituted catalase tubes (DMS alk) were prepared as described in section 3.2. In each case the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. The stabilities of the three derivatives were assessed in the manner described in section 3.7 and are shown in Fig 16. The HMDA-substituted catalase tube exhibited poor stability, being completely deactivated after 20 h whilst the protein- and denatured-protein-substituted derivatives retained 36% and 52% of their original activities respectively. The last derivative therefore exhibited greatest stability. This may be due to the higher activity of this derivative thus depleting the H_2O_2 concentration more rapidly. These results therefore suggest that maximum stability is achieved on immobilisation of large quantities of enzyme.

4.2.4. Stability of Immobilised Catalase (DMS alk) from Different Sources

The results of the previous sections indicated the instability of beef-liver catalase when immobilised upon DMS-alkylated nylon-tubes. Scott and Hammer (53) have compared the relative stabilities of soluble catalase from mammalian and fungal sources and found the latter to exhibit greater stability to pH extremes and increased resistance to substrate deactivation.

The relative stabilities of the two nylon-tube immobilised enzymes were studied in the following manner. A 6 m length of glutaraldehyde activated denatured protein-substituted nylon-tube was prepared as described in section 3.2. One 3 m length was filled with a 1.4 mg ml^{-1} solution of beef-liver catalase and the other with a 1.4 mg ml^{-1} solution

Fig 17 . Stability of nylon-tube immobilised catalase derivatives (DMS-alk.) from beef-liver and fungal sources to continuous perfusion with 1.5 mM-H_2O_2 at pH 6.9 . ● immobilised beef-liver catalase ; ▣ immobilised fungal catalase .


1.5
1.0
0.5
Ln [H2O2]° / [H2O2]t
30
60
90
Time (h)

of catalase from Aspergillus Niger. After the coupling and washing procedure described in section 3.2.4 the derivatives were assayed for bound protein and catalatic activity. In each case 33% of the total protein was coupled and the beef-liver and fungal enzyme derivatives had catalatic activities of 13 x 10^{-3} cm^2 min^{-1} and 7 x 10^{-3} cm^2 min^{-1} respectively. Thus the immobilised fungal catalase displayed 54% of the activity of the beef-liver catalase derivative. This ratio corresponds to the relative specific activities of the commercial preparations used in the coupling procedure, (15,000 and 9,000 IU).

The two derivatives were subjected to continuous perfusion with 1.5 mM-H_2O_2 as described in section 3.7. The results are shown in Fig 17. After 34 h of perfusion the beef-liver catalase derivative had lost all of its activity, whereas the fungal derivative still retained 80% of its original activity. Thus the stability of nylon-tube immobilised catalase (DMS alk) is improved with the use of fungal catalase. Recently, Altmore et al. (20) have demonstrated similar improvements in stability of fungal catalase bound to silica alumina particles.

4.3. STABILITY OF NYLON-TUBE IMMOBILISED CATALASE PREPARED BY TOTFB-ALKYLATION

4.3.1. Stability to Continuous Perfusion of 1.5 mM-H_2O_2.

The stability of nylon-tube immobilised catalase derivatives at pH 6.9 was studied in the following manner. A 1.5 m length of HMDA-substituted catalase tube was prepared as described in section 3.2. The enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. The derivative was subjected to continuous perfusion of 1.5 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9, as described in section 3.7. No loss in activity occurred over a two week period. This result was confirmed by

assaying the derivative before and after perfusion with H_2O_2, using the recirculation tecnique described in section 3.5.4.

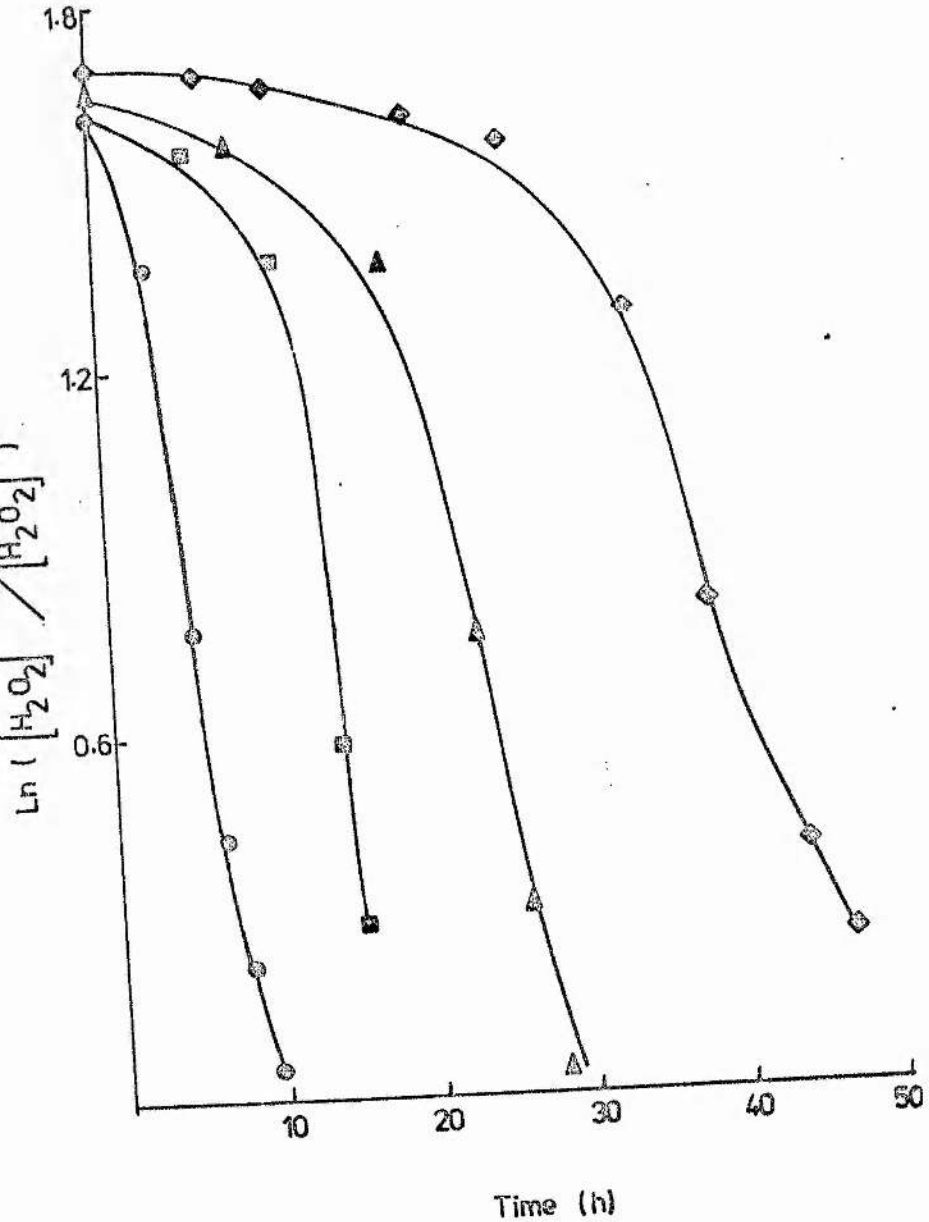
The stability of nylon-tube immobilised catalase prepared by TOTFB alkylation of the nylon is therefore far greater than that obtained with DMS-alkylation, the latter derivative becoming inactive after only 22 h exposure to the same conditions as shown in section 4.2.3. The reasons for this stabilisation effect may be found by consideration of the differing quantities of enzyme bound to the two differently alkylated supports. It has already been suggested in section 4.2.3 that an increase in the activity of bound protein may stabilise the enzyme by increasing the H_2O_2 concentration gradient existing across the enzyme tube due to substrate utilisation. TOTFB alkylation has been shown in section 4.1.6 to result in a 63% increase in bound protein when compared with derivatives prepared with DMS alkylation.

4.3.2. Bound Protein Stabilisation Effect of Catalase Tubes

The bound protein stabilisation effect was further studied in the following manner. A 6 m length of activated HMDA-substituted nylon-tube was prepared as described in section 3.2. The derivative was divided into four 1.5 m lengths, filled with enzyme coupling solutions comprising 0.25 mg ml^{-1}, 0.5 mg ml^{-1}, 1.0 mg ml^{-1}, and 4.0 mg ml^{-1} catalase respectively, and then submitted to identical coupling and washi procedures as described in section 3.2.4. The amount of bound protein was estimated in the manner described in section 3.3. The derivatives were then perfused with 10 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9, at 25°C and a flow-rate of 2.9 ml min^{-1}. The stability of each derivative was monitored in the manner described in section 3.6.1 and the results are shown in Fig 18. The derivatives were found to immobilise 100% of the

Fig 18 . Dependence of the stability of nylon-tube.immobilised catalase derivatives upon the bound protein content of the tube.

● 0.26 mg ; ■ 0.52 mg ; ▲ 1.04 mg ; ◆ 4.1 mg catalase bound to 1.5 m glutaraldehyde activated/HMDA-substituted nylon-tube.

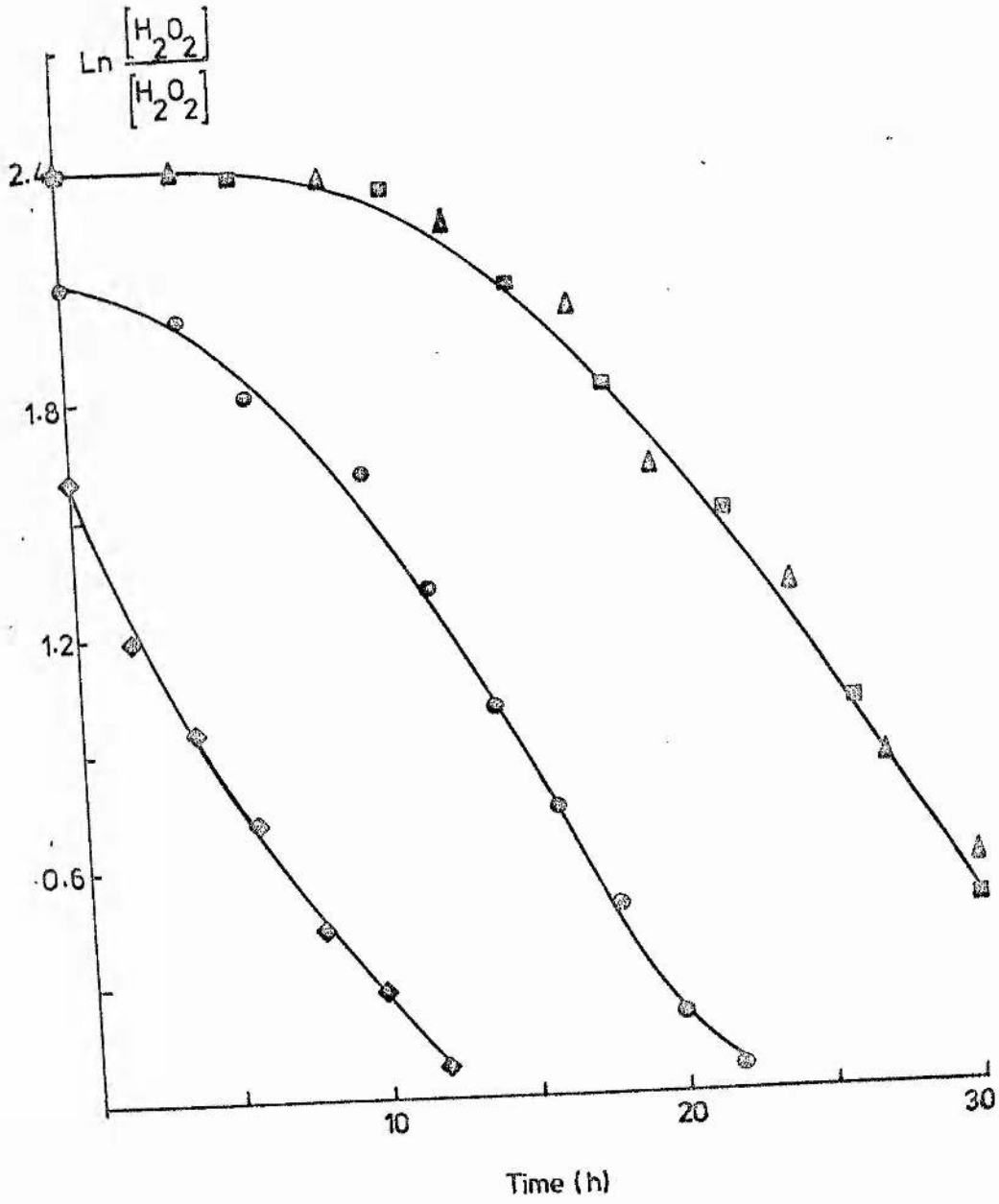

1·8
1·2
0.6
Ln ([H2O2]° / [H2O2]t)
10
20
30
40
50
Time (h)

available catalase and so consisted of 0.26 mg, 0.52 mg 1.04 mg and 4.1 mg catalase bound to 1.5 m of the substituted nylon-tube. The catalatic activity of each derivative was identical when assayed by the method described in section 3.5.4. These derivatives lost 90% of their original activities after 9 h, 16 h, 28 h, and 50 h respectively. Thus the stability of nylon-tube immobilised catalase to H_2O_2 at pH 6.9 increases with the bound protein content. The reasons for this are not clear. In some way, the presence of 'inactive' immobilised catalase inhibits the rate of substrate deactivation of the active enzyme.

4.3.3. Effect of Spacers on Stability of Catalase Tubes

The effect of spacers upon the stability of nylon-tube immobilised catalase derivatives was studied in the following manner. 1.5 m lengths of HMDA-, adipic acid dihydrazide-, and methylated egg-albumen-substituted catalase tubes were prepared as described in section 3.2. In each case the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. The enzyme tubes were submitted to continuous perfusion with 10 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9, as described in section 3.6.1. The results depicted in Fig 19, show that the adipic acid dihydrazide-substituted catalase derivative lost 90% of its activity over 20 h whilst the HMDA- and methylated egg-albumen substituted derivatives retained 70% of their activities over a similar period. As shown in Table 4, substitution of nylon-tube with adipic acid dihydrazide in place of HMDA results in a 53% drop in bound protein. This result is therefore consistent with the protein stabilisation effect reported in the previous section. The use of a protein spacer in place of HMDA did not increase the stability of the immobilised enzyme as it did with DMS-alkylated catalase tubes. However, the larger amounts

Fig 19 . Effect of spacers upon the stability of nylon-tube immobilised catalase derivatives to perfusion with 10 mM-H_2O_2 at pH 6.9 . ▣ HMDA-substituted ; ○ Adipic acid dihydrazide-substituted ; △ Methylated egg albumen substituted nylon-tube immobilised catalase. ◈ stability of an adipic acid dihydrazide-substituted catalase derivative in the presence of 0.5 M-NaCl.


Ln [H2O2] / [H2O2]
2.4
1.8
1.2
0.6
10
20
30
Time (h)

of enzyme immobilised upon TOTFB-alkylated nylon-tube already stabilise the bound enzyme. This effect will tend to decrease the importance of any spacer stabilisation.

4.3.4. Effect of Salt on the Stability of Catalase Tubes

The presence of salt is known to decrease the stability of soluble catalase by enhancing the formation of the so-called compound 2 (54). The effect of salt upon the nylon-tube immobilised enzyme was studied in the following manner. A 1.5 m length of adipic acid dihydrazide-substituted catalase tube was perfused with 10 mM-H_2O_2 at pH 6.9 as described in section 3.6.1. An identical derivative was then perfused with 10 mM-H_2O_2 in the presence of 0.5 M-NaCl under identical conditions. The stabilities of the catalase derivatives in the presence and absence of salt are depicted in Fig 19. In the presence of salt, the initial activity dropped by 23% and deactivation occurred over a 13 h period. In the absence of salt, deactivation occurred over 23 h. Thus 0.5 M-NaCl adversely affects the stability of immobilised catalase, as well as that of the soluble enzyme.

4.3.5. Stability of Enzyme from Different Sources

The effect of the source of catalase on the stability of the nylon-tube immobilised enzyme was determined in the following manner. Beef-liver and fungal catalase were immobilised onto separate 1.5 m lengths of activated, HMDA-substituted catalase tube. In each case the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. Each derivative was perfused with 10 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9, as described in section 3.6.1. The stabilities of the derivatives are depicted in Fig 21. The beef-liver immobilised catalase lost 80% of its activity over a period of 29 h whilst the fungal enzyme lost only 11%. Thus at

Fig 21 . Stability of nylon-tube immobilised catalase (DMS treated) derivatives from beef-liver and fungal sources to continuous perfusion with 10 mM-H_2O_2 at pH 6.9 . ● immobilised beef-liver catalase ; ■ immobilised fungal catalase.

Fig 22 . Stability of nylon-tube immobilised catalase (DMS treated) derivatives from beef-liver and fungal sources to continuous perfusion with 1.0 mM-H_2O_2 at pH 8.5 . ● immobilised beef-liver catalase ; ■ immobilised fungal catalase.

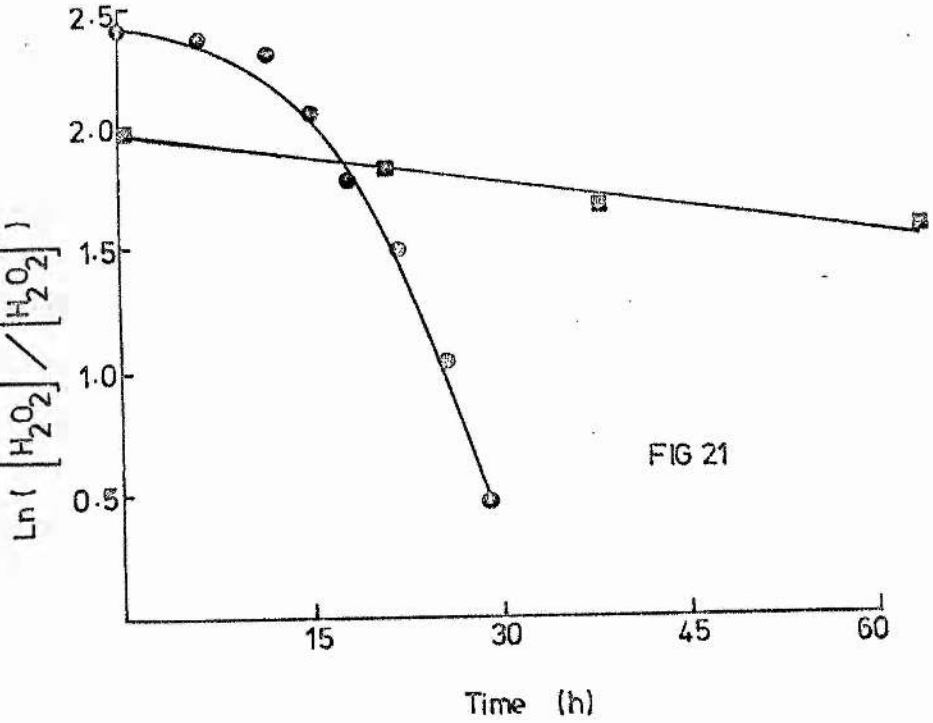

2·5
2·0
1·5
1·0
0·5
Ln ([H2O2]° / [H2O2]t)
15
30
45
60
Time (h)

FIG 21

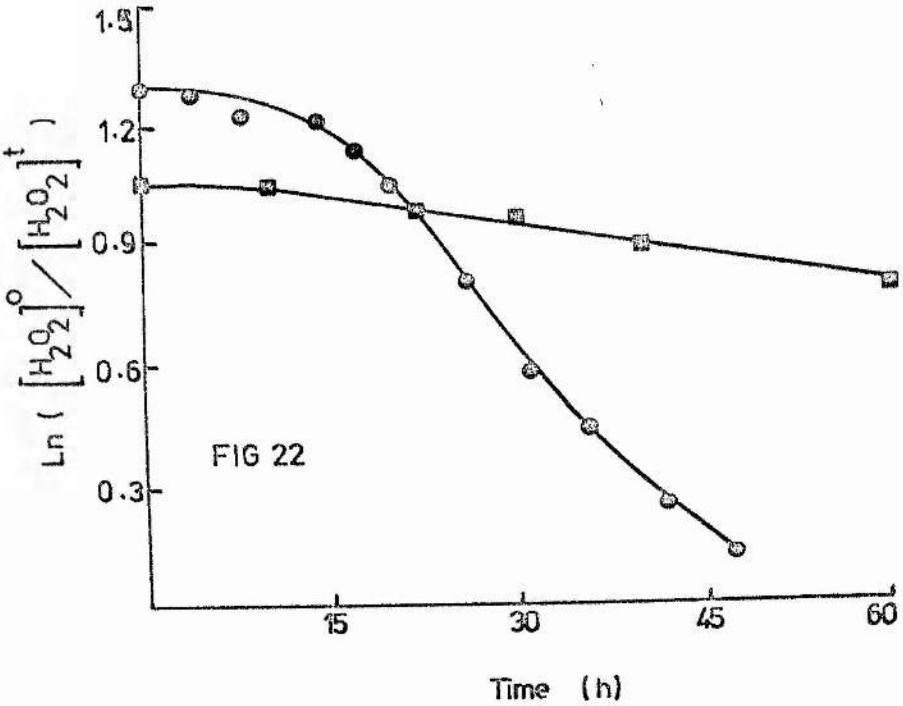

1·5
1·2
0·9
0·6
0·3
Ln ([H2O2]° / [H2O2]t)
15
30
45
60
Time (h)

FIG 22

pH 6.9, the stability of the fungal catalase was superior to that of the beef-liver derivative.

The stability of each derivative at alkaline pH was also determined. 1.0 mM-H_2O_2 in 0.2 M-borate, pH 8.5 was perfused through each derivative at 25°C and a flow rate of 2.9 ml min^{-1}. Whereas no deactivation of either derivative occurred at pH 6.9, the results, depicted in Fig 22, show that the beef-liver and fungal immobilised enzymes lost 75% and 15% of their original activity respectively at pH 8.5 over a 40 h period. Thus although both enzymes were less stable at alkaline pH, the fungal enzyme again exhibited better stability than the beef-liver enzyme.

SUMMARY OF SECTIONS 4.2. AND 4.3.

The stability of several nylon-tube immobilised catalase derivatives has been discussed. In general, DMS-alkylated derivatives were found to be unstable at pH 6.9 in the presence of 1.5 mM-H_2O_2. This was explained by a substrate effect rather than inherent instability of the immobilised enzyme. The use of protein spacers produced the most stable DMS-alkylated catalase tubes. However, in the case of a glutaraldehyde activated/ denatured protein substituted/DMS-alkylated catalase tube, a 48% decrease in activity was observed over 20 h of exposure to 1.5 mM-H_2O_2 at pH 6.9 and 25°C. The use of catalase from a fungal origin further improved the stability of the immobilised derivative, 50% losses in activity being observed over 78 h of exposure to identical conditions as described above.

However, greater improvements in stability were observed on using TOTFB as the alkylation reagent in the preparation of catalase tubes. A glutaraldehyde activated/ HMDA-substituted/ TOTFB alkylated catalase tube exhibited complete stability on exposure to 1.5 mM-H_2O_2 over a

period of two weeks. Exposure of the derivative to 10 mM-H_2O_2 caused losses in activity to occur, although the immobilisation of larger quantities of enzyme was observed to stabilise the derivative in some manner. The smaller amounts of enzyme bound to dihydrazide-substituted derivatives adversely affected the stability of these derivatives when compared with HMDA-substituted catalase tubes.

The use of catalase of fungal origin again improved the stability of the immobilised catalase such that only a 11% decrease in activity was observed over a 29 h period of exposure to 10 mM-H_2O_2 at pH 6.9, whereas a derivative prepared using catalase from beef-liver lost 80% of its activity during this time. Use of the fungal enzyme also improved the stability of nylon-tube immobilised catalase derivatives at pH 8.5.

Thus a glutaraldehyde activated/HMDA-substituted/TOTFB-alkylated nylon-tube immobilised fungal catalase derivative was shown to exhibit the greatest stability.

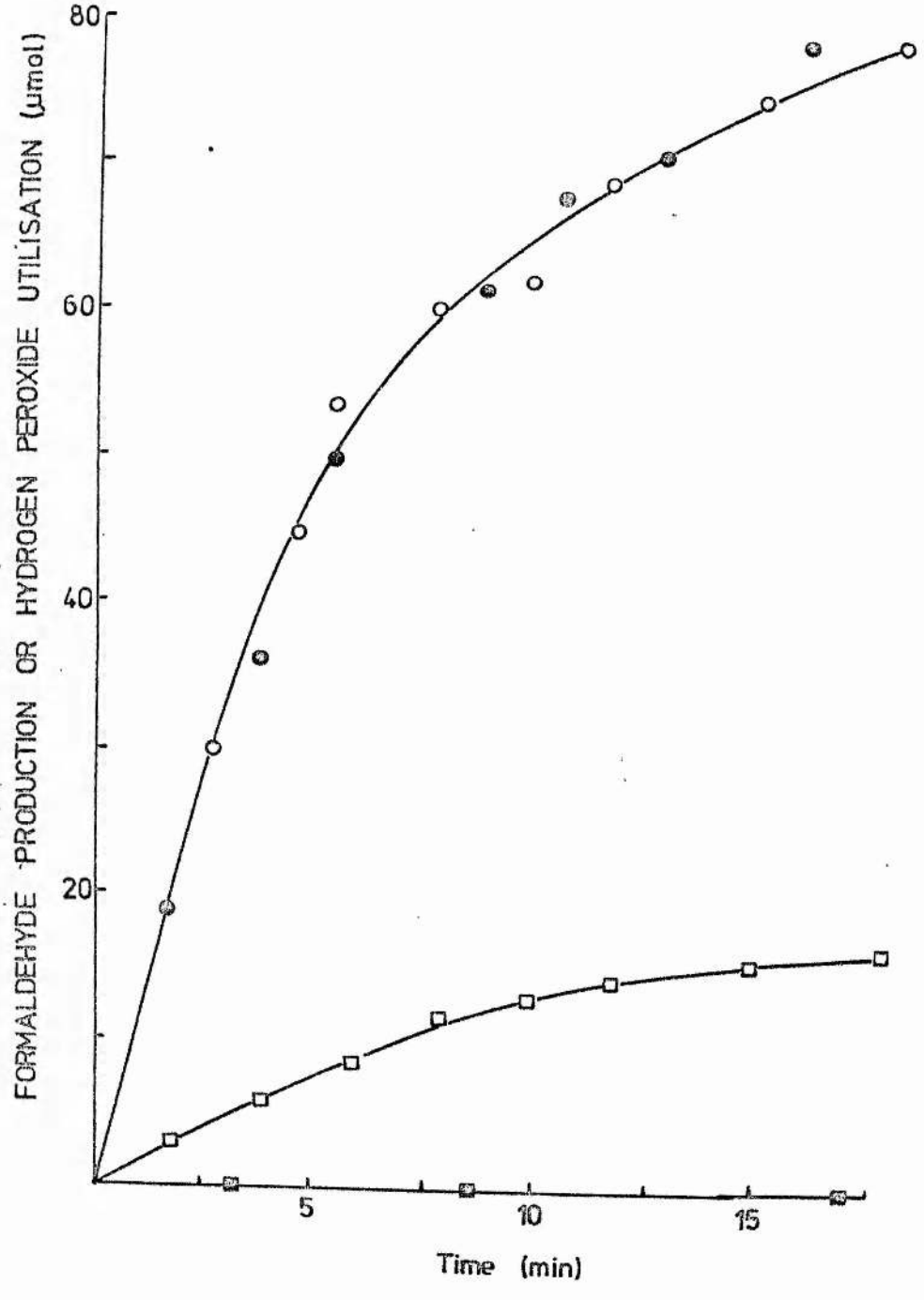
4.4. PEROXIDATIC ACTIVITY OF NYLON-TUBE IMMOBILISED CATALASE

4.4.1. The Peroxidatic Activity of Catalase Tubes

Soluble catalase has been shown to exhibit peroxidatic activity in the presence of H_2O_2 and methanol or ethanol (55-58). The catalatic and peroxidatic reactions exist in competition between each other without increasing the overall rate of H_2O_2 utilisation (58). The peroxidatic activity of an immobilised catalase derivative was investigated in the following manner. A 2 m length of HMDA-substituted catalase tube (DMS alk) was assayed for catalatic and peroxidatic activities in the presence and absence of methanol as described in sections 3.5.4 and

Fig 23 . Peroxidatic activity of nylon-tube immobilised catalase in the presence and absence of 2.5 M-methanol.

○ H_2O_2 utilisation in the presence of methanol ; ◎ H_2O_2 utilisation in the absence of methanol ; □ HCHO production in the presence of methanol ; ■ HCHO production in the absence of methanol.


FORMALDEHYDE PRODUCTION OR HYDROGEN PEROXIDE UTILISATION (µmol)
80
60
40
20
5
10
15
Time (min)

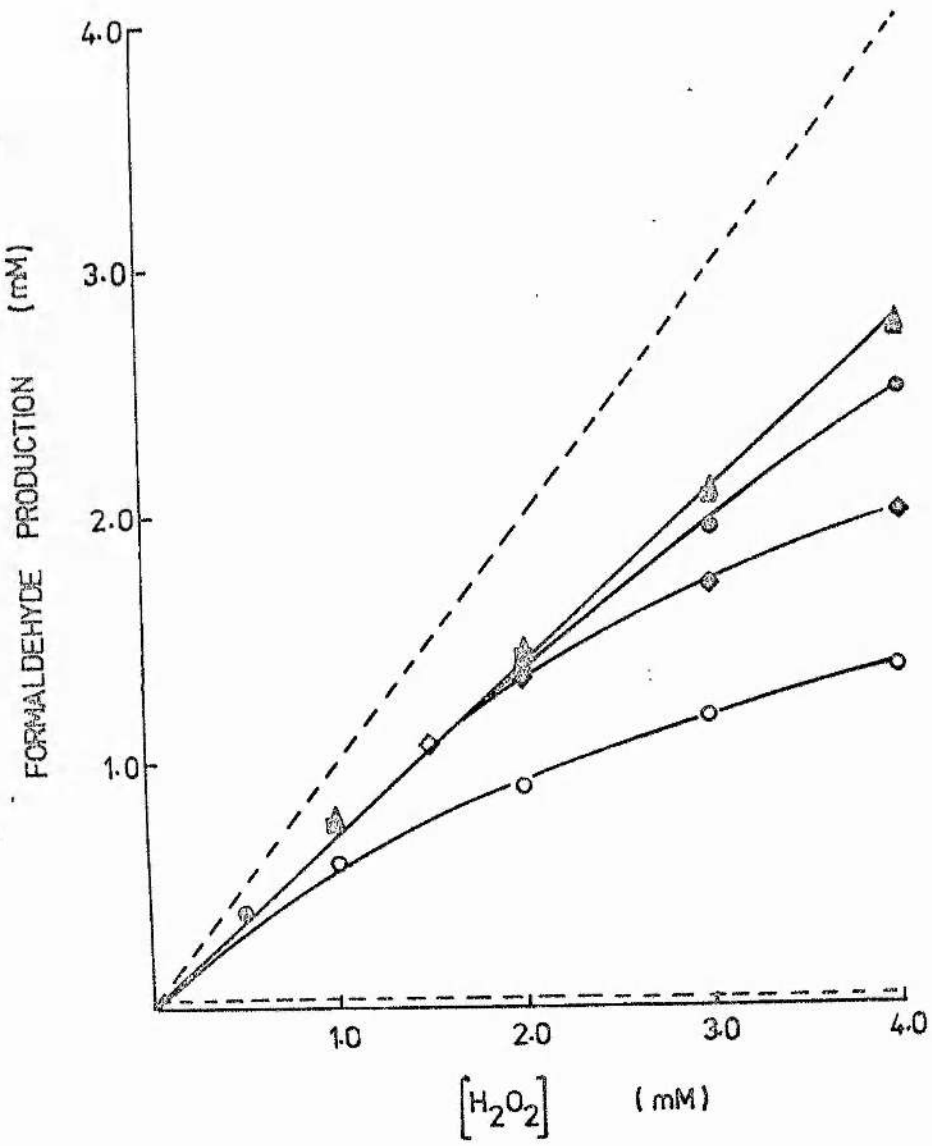
3.5.5. In this assay, both the emergence of HCHO and the utilisation of H_2O_2 were monitored. The results are shown in Fig 23. The rate of utilisation of H_2O_2 was identical in the presence and absence of methanol, but HCHO was produced only in the former case. The sum of the peroxidatic and catalatic activities of the derivative in the presence of methanol was equal to the catalatic activity in the absence of methanol. This observation suggests that, as in the soluble system, competition occurs between the two catalytic functions of catalase. The efficiency of the peroxidatic conversion was low, only 20% of the H_2O_2 utilised being converted to HCHO. The assay conditions therefore seem to favour the catalatic action of the immobilised enzyme.

4.4.2. Effect of Methanol Concentration on Peroxidatic Activity of Catalase Tubes

The effect of different methanol concentrations on the peroxidatic activity of nylon-tube immobilised catalase was determined in the following manner. A 2 m length of HMDA-substituted catalase tube was prepared as described in section 3.2. It was then perfused with 1 mM- and 4 mM-H_2O_2 in 0.05 M-phosphate, pH 6.9, at 25°C and a flow rate of 1.4 ml min^{-1}. In each case the tube effluent was analysed for H_2O_2 by the acid-KI tecnique described in section 3.7. 97% conversion of the H_2O_2 was found to occur under these conditions.

The peroxidatic activity of the derivative was then measured in the manner described in section 3.6.4. The concentration of alcohol in the 0.05 M-phosphate, pH 6.9, was varied between 0.25 M and 3.0 M-methanol. 1 mM- 4 mM- H_2O_2 samples were assayed and the Hantzsch reaction checked for susceptibility to variation in methanol concentration by standardisation with HCHO at all concentrations of methanol employed. The results are shown in Fig 24.

Fig 24 . Effect of methanol concentration on the peroxidatic activity of nylon-tube immobilised catalase. ○ 0.25 M ; ◈ 0.5 M ; ◉ 1.5 M ; ▣ 2.5 M ; Δ 3.0 M-methanol. Dashed lines represent 100% and 0% peroxidatic utilisation of H_2O_2.

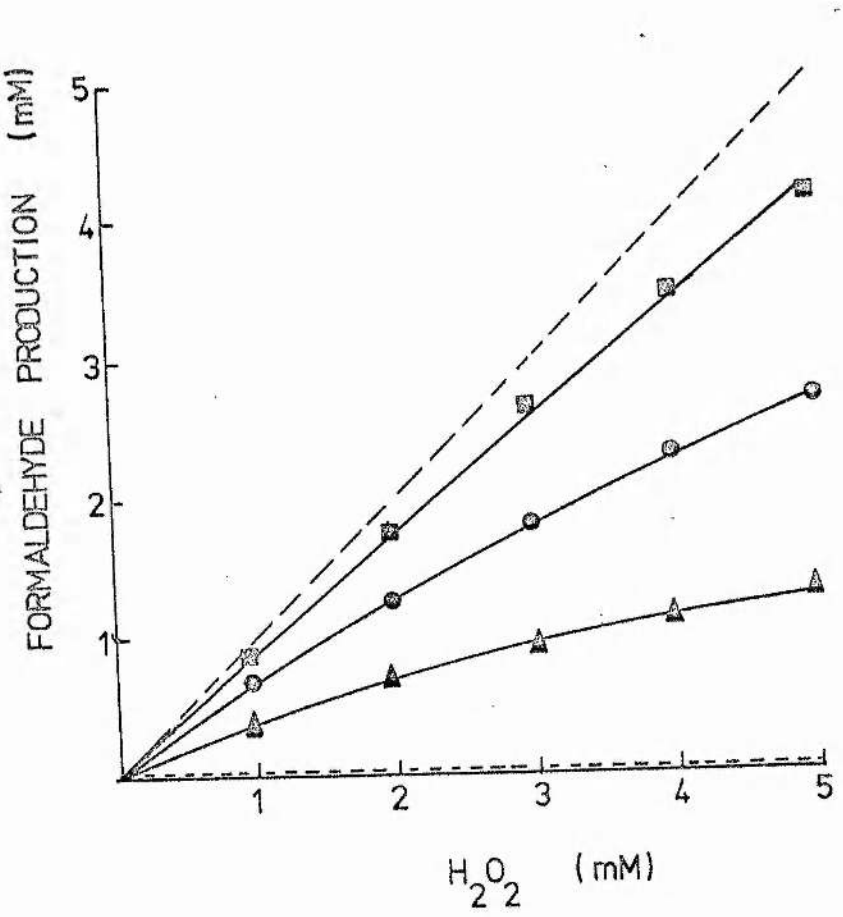

4.0
3.0
2.0
1.0
FORMALDEHYDE PRODUCTION (mM)
1.0
2.0
3.0
4.0
[H2O2] (mM)

In the presence of 2.5 M-methanol, 75% of each H_2O_2 sample was used peroxidatically in the production of HCHO. As all the substrate was utilised by the catalase derivative, the remaining 25% H_2O_2 was presumably converted to oxygen and water via the catalatic reaction. Higher concentrations of methanol showed no increase in the efficiency of the peroxidatic activity, whilst decreasing concentrations resulted in corresponding decreases in the peroxidatic : catalatic ratio. For example in the presence of 0.25 M-methanol only 60% of 1 mM-H_2O_2 and 35% of 4 mM-H_2O_2 samples were converted peroxidatically. Thus the presence of 2.5 M-methanol is required for maximal peroxidatic activity of catalase tubes.

4.4.3. Effect of Immobilisation Chemistry and Tube Diameter on Peroxidatic Activity of Catalase Tubes

The effect of different alkylation processes and tube diameter on the peroxidatic activity of nylon-tube immobilised catalase was studied in the following manner. Three 2 m lengths of HMDA-substituted catalase tubes were prepared in the following way : 1) by DMS-alkylation of 1 mm bore nylon-tube, 2) TOTFB alkylation of 1 mm bore nylon-tube, 3) TOTFB alkylation of 2 mm bore nylon-tube. In each case the enzyme coupling solution comprised 1.4 mg ml^{-1} catalase. The efficiency of the peroxidatic activity of each derivative was determined by the method described in section 3.6.4. 1-5 mM-H_2O_2 samples were assayed in the presence of 2.5 M-methanol at pH 6.9, and the system calibrated with standardised HCHO samples. The results are shown in Fig 25. The upper and lower dashed lines are theoretical plots representing 100% peroxidatic and 100% catalatic actions respectively. As total conversion of H_2O_2 was attained, plots lying between the two extremes indicate the occurrence of both activities. It can be observed that the 2mm bore derivative most closely approached 100% peroxidatic activity, whilst the

Fig 25 . Peroxidatic activity associated with nylon-tube immobilised catalase derivatives. Dashed lines are theoretical plots representing 100% and 0% peroxidatic utilisation of H_2O_2. ▲ 1 mm bore nylon-tube immobilised catalase (DMS-alk.) ; ● 1 mm bore nylon-tube immobilised catalase (TOTFB-alk.) ; ■ 2 mm bore nylon-tube immobilised catalase (TOTFB-alk.).


FORMALDEHYDE PRODUCTION (mM)
5
4
3
2
1
1
2
3
4
5
H_2O_2 (mM)

1 mm bore DMS-alkylated derivative displayed mostly catalatic behaviour. This can be explained by the differing quantities of active enzyme bound to the supports. It has previously been shown in section 4.1 that the enzymic activity of immobilised derivatives decreases in the following sequence :
TOTFB alk, 2 mm bore catalase tubes > TOTFB alk, 1 mm bore catalase tubes > DMS alk 1 mm bore catalase tubes. In the case of the soluble enzyme it has been found that the ratio of the peroxidatic : catalatic activities increases with increased enzyme concentration (59). Thus the same effect seems to be occurring for the immobilised derivatives.

4.4.4. Effect of Tube Length on Peroxidatic Activity

The dependence of the peroxidatic : catalatic activity ratio on bound enzyme quantity, prompted an investigation into the variation of this ratio with tube length. A 1.5 m length of HMDA-substituted 2 mm bore catalase-tube was prepared as described in Section 3.2. The peroxidatic activity associated with 0.25 m, 0.5 m, 1.0 m, and 1.5 m lengths of this derivative was determined in the manner described in section 3.6.4. The results, depicted in Fig 26 show that whilst a linear response was obtained for both the 1.5 m and 1.0 m lengths, the response of the two shorter tubes plateaued at higher H_2O_2 concentrations. This result can again be explained by competition between the two catalytic activities of the enzyme. Normally, increased concentrations of H_2O_2 favor the catalatic action. However, the presence of large quantities of bound enzyme in the 1.0 m and 1.5 m catalase tubes appears to negate this effect. In the shorter tube lengths, the lower quantity of bound enzyme is insufficient to overcome the effect, and the catalatic activity therefore increases.

Fig 26 . Dependence of peroxidatic activity upon the length of nylon-tube immobilised catalase. ◆ 0.25 m ; ● 0.5 m ; ■ 1.0 m ; ▲ 1.5 m of catalase tube.

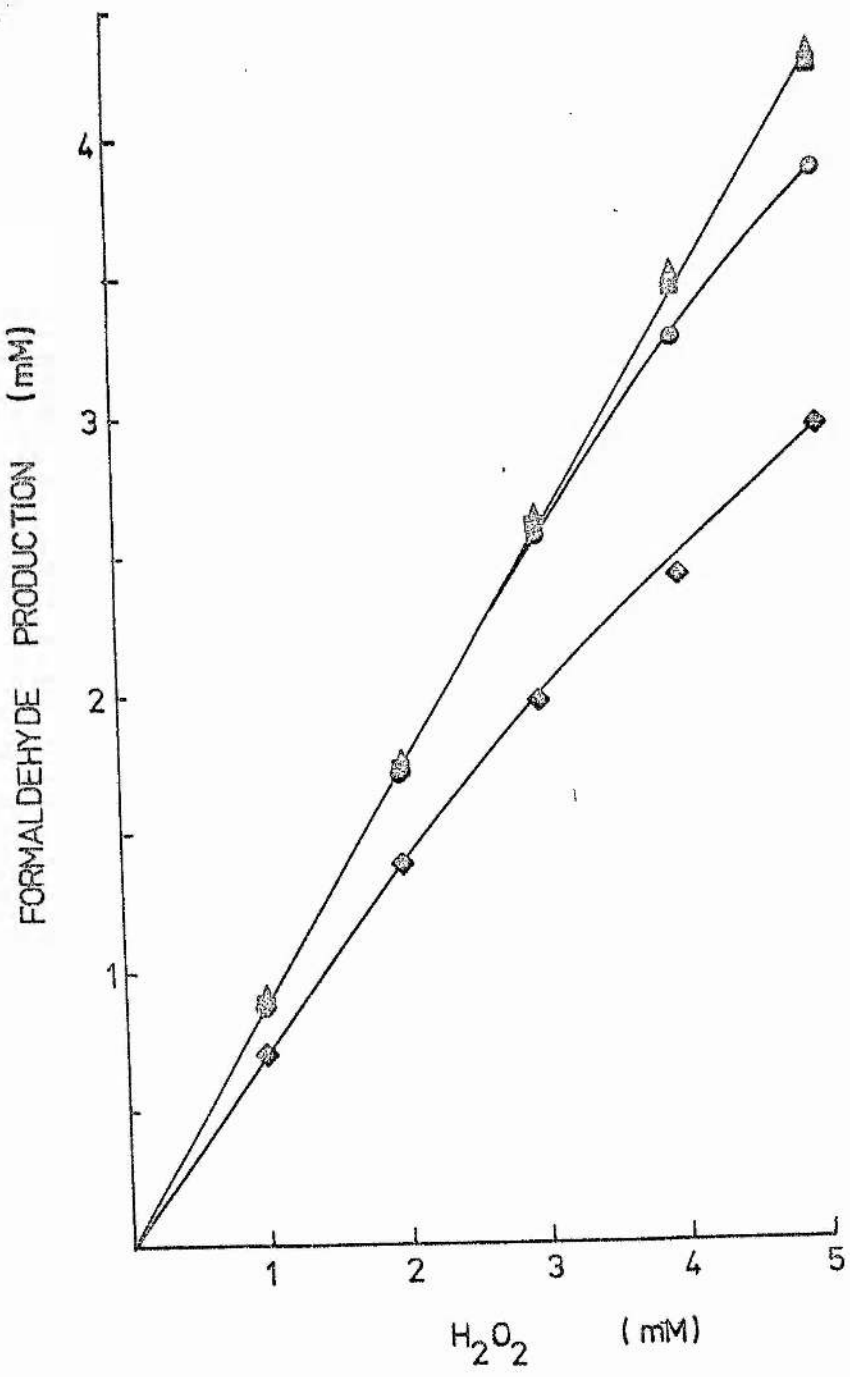
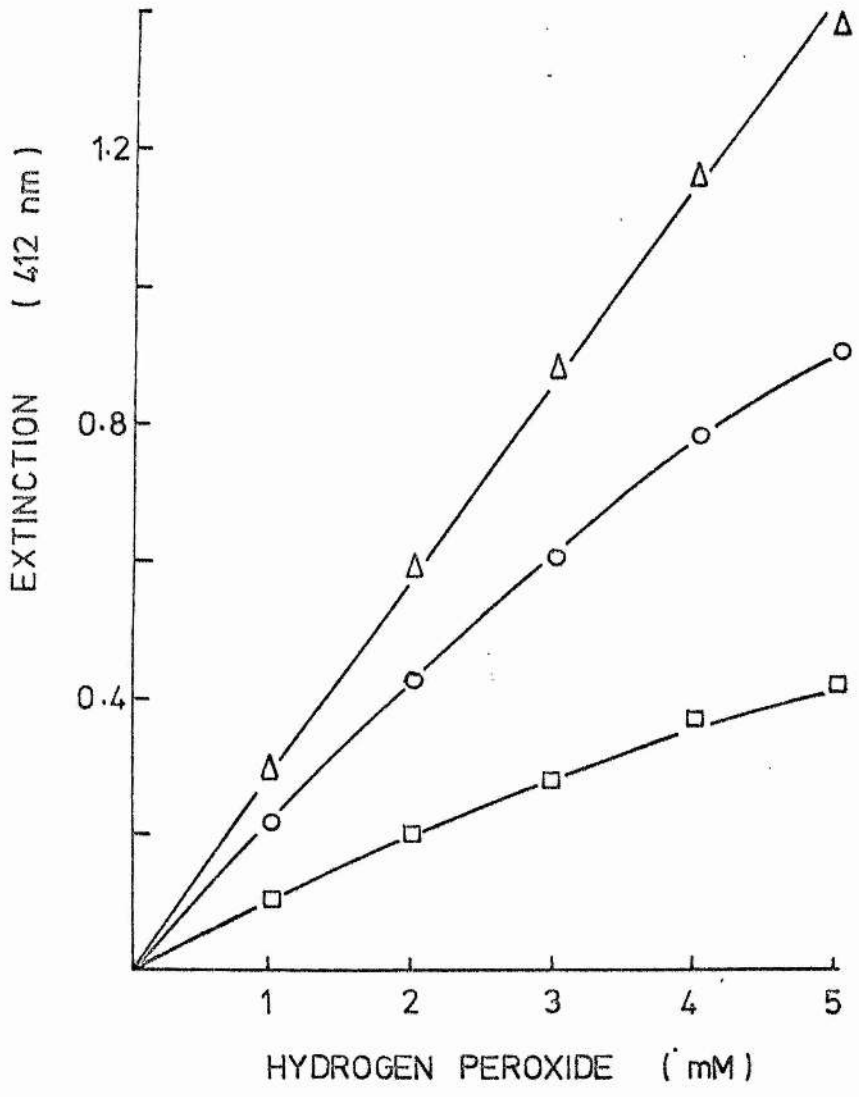

FORMALDEHYDE PRODUCTION (mM)
4
3
2
1
1
2
3
4
5
H_2O_2 (mM)

Fig 27 . Standard curve for the estimation of H_2O_2 using 2 m lengths of : Δ 2 mm bore nylon-tube immobilised catalase (TOTFB-alk.)

○ 1 mm bore nylon-tube immobilised catalase (TOTFB-alk.)

□ 1 mm bore nylon-tube immobilised catalase (DMS-alk)


EXTINCTION (412 nm)
1·2
0·8
0·4
1
2
3
4
5
HYDROGEN PEROXIDE (mM)

4.4.5. Comparison of the Peroxidatic Activity of Beef-liver and Fungal Catalase Tubes

The peroxidatic activities of immobilised preparations of both beef-liver and fungal catalase were compared in the following manner. Two 3 m lengths of activated HMDA-substituted nylon-tube were prepared as described in section 3.2. One such tube derivative was incubated with an enzyme coupling solution comprising 1.4 mg ml^{-1} beef-liver enzyme and the other with 1.4 mg ml^{-1} fungal enzyme. After washing, each derivative was assayed for peroxidatic activity as described in section 3.6.4. No difference in the rate of HCHO production of either derivative could be detected. Thus in each case there must be sufficient immobilised activity to attain the highest ratio of peroxidatic : catalatic activity.

It has been shown in section 4.3.5 that the fungal enzyme displays superior stability. As the peroxidatic activities of the fungal and beef-liver immobilised derivatives are identical, the former may therefore be more useful as an insoluble enzyme for autoanalytical work.

4.4.6. Standard Curves for the Determination of H_2O_2

H_2O_2 was determined by the peroxidatic reaction of immobilised catalase as described in section 3.6.4. Three HMDA-substituted derivatives were prepared in the following way : 1) DMS alkylation of 1mm bore tube 2) TOTFB alkylation of 1mm bore tube, 3) TOTFB alkylation of 2 mm bore tube. Each was inserted into the flow system described in Fig 86 and standardised solutions of 1mM - 5mM-H_2O_2 were sampled. The standard curves obtained for the three catalase derivatives are shown in Fig 27. The highest sensitivity was obtained with the TOTFB alkylated 2 mm bore catalase tube, whilst the 1 mm bore DMS alkylated catalase tube displayed the lowest sensitivity. This demonstrates the

superiority of the activity of the wide bore catalase tubes for autoanalytical work.

4.4.7. Stability of Catalase Tubes to the Peroxidatic Action

The stability of catalase tubes working in the catalatic mode have previously been discussed in section 4.3. However, a catalase tube working in the peroxidatic mode is exposed to methanol and HCHO in addition to H_2O_2 and buffer. In order to determine the operational stability of catalase tubes under these conditions, the following experiment was undertaken.

Two 1.5 m lengths of HMDA-substituted nylon-tube immobilised catalase were prepared from beef-liver and fungal enzyme, as described in section 3.2. Both derivatives were subjected to continuous analysis of 5 mM-H_2O_2 as described in section 3.6.4. Each derivative was found to be stable over a period of 60 h over which 3600 assays were performed suggesting that no decrease in stability of a catalase tube is caused by exposure to conditions imposed by the peroxidatic conversion of methanol to formaldehyde.

SUMMARY OF SECTION 4.4

The peroxidatic and catalatic activities of immobilised catalase were investigated. The sum of the combined activities in the presence of both H_2O_2 and methanol was found to equal the catalatic activity in the absence of methanol. This suggested that competition exists between the peroxidatic and catalatic activities. The presence of 2.5 M-methanol gave the highest ratio of peroxidatic : catalatic activity displayed by the immobilised derivative. The extent of peroxidatic activity associated with different immobilised catalase tubes was investigated. TOTFB-alkylation was found to produce a

derivative in which the ratio of peroxidatic : catalatic activity was higher than a derivative prepared by DMS-alkylation. Increasing the diameter of the tube from 1 - 2 mm also increased this ratio. The length of catalase tube assayed was shown to affect the extent of peroxidatic utilisation of the H_2O_2. A linear response of formaldehyde production with hydrogen peroxide concentration was obtained using a 1.5 m length of glutaraldehyde activated/ HMDA-substituted/ TOTFB-alkylated 2 mm-bore/ nylon-tube immobilised catalase, while a non-linear response was obtained with shorter lengths. The peroxidatic activity of the fungal enzyme was also examined and found to utilise 80% of the H_2O_2 in a peroxidatic manner when immobilised to a HMDA-substituted/ TOTFB-alkylated/ 2 mm-bore/ nylon-tube.

Thus for autoanalytical use the derivative displaying the greatest peroxidatic activity was glutaraldehyde activated/ HMDA-substituted/ TOTFB-alkylated 2 mm-bore/nylon-tube immobilised fungal catalase.

The sensitivity of an autoanalytical system for the determination of H_2O_2 using TOTFB-alkylated nylon tube derivatives of catalase was found to be adequate.

CHAPTER 5

DETERMINATION OF GLUCOSE USING NYLON-TUBE IMMOBILISED GLUCOSE OXIDASE IN CONJUNCTION WITH NYLON-TUBE IMMOBILISED CATALASE

The peroxidatic activity of catalase which was discussed in the previous section, can be utilised, in conjunction with glucose oxidase, in the determination of glucose. In one assay system, the H_2O_2 which is produced by the primary glucose oxidase reaction is used in the peroxidatic conversion of methanol to formaldehyde by catalase. The formaldehyde thus produced is quantitively measured by means of the Hantzsch reaction. The complete reaction sequence for this assay is schematically represented in Fig 9.

Alternatively, the H_2O_2 produced by the glucose oxidase reaction can be used for the peroxidatic conversion of ethanol to acetaldehyde by catalase. The acetaldehyde thus produced may then be monitored by a third enzyme, aldehyde dehydrogenase. This enzyme oxidises acetaldehyde to acetate using the coenzyme NAD^+. Thus spectrophotometric monitoring of the formation of NADH affords a convenient means of measuring H_2O_2 and thus glucose.

A third "reagentless" assay protocol for the determination of glucose using nylon-tube coimmobilised derivatives of glucose oxidase and catalase may be designed. In this assay, the oxygen utilisation of the glucose reaction is monitored with a flow-through oxygen electrode. The presence of excess coimmobilised catalase, although replacing half of the oxygen used by the former reaction will ensure the absence of errors arising from endogenous catalase.

5.1. DETERMINATION OF GLUCOSE USING NYLON-TUBE COIMMOBILISED GLUCOSE OXIDASE AND CATALASE

In a clinical analytical system incorporating nylon-tube immobilised enzymes, the preparation of the derivative is of prime importance. It has been shown in chapter 4 that the use of different methods for the covalent linking of catalase to nylon-tubes produces marked

differences in the amount of immobilised activity retained by the derivative. In any assay system it is clearly preferable to attain maximal sensitively. In the case of assays involving nylon-tube immobilised enzymes this requires the preparation of a derivative by the methods that produce greatest activity.

The effect of different alkylating reagents, different spacers, different ratios of the two enzymes in the coupling solution and different tube diameters, on the combined activity of glucose oxidase and catalase co-immobilised to the same nylon-tube was studied in order to produce the derivative best suited for analytical use.

5.1.1. Preparation of Nylon-Tube Co-immobilised Glucose Oxidase and Catalase

It was previously shown in section 4.4.3 that only a minority of the H_2O_2 utilised by a DMS-alkylated catalase tube was involved in the peroxidatic conversion of methanol to formaldehyde. Keilin and Hartree (60) have shown that the ratio of peroxidatic : catalatic activity of soluble catalase is low under conditions where the reaction is initiated in the presence of large concentrations of H_2O_2, and high under conditions where the H_2O_2 is slowly and continually generated, for example by a subsidiary enzyme system such as glucose oxidase. This implies that a higher percentage of H_2O_2 produced from the glucose oxidase reaction would be peroxidatically utilised by catalase if the two enzymes were co-immobilised upon the same support. These conditions should result in a situation where the H_2O_2 produced by the glucose oxidase reaction could be instantly utilised by adjacently immobilised catalase, thus ensuring the absence of high concentrations of H_2O_2 in the system.

The use of nylon-tube co-immobilised glucose oxidase and catalase (subsequently referred to as GOD/CAT tubes) in the determination of

Fig 28 . Standard curve for the estimation of glucose using GOD/CAT derivatives. O TOTFB-alkylated derivative ; □ DMS-alkylated derivative.

Fig 29 . Effect of different spacers on the combined activity of GOD/CAT derivatives. O HMDA ; □ Adipic acid dihydrazide ; ● Succinic acid dihydrazide.

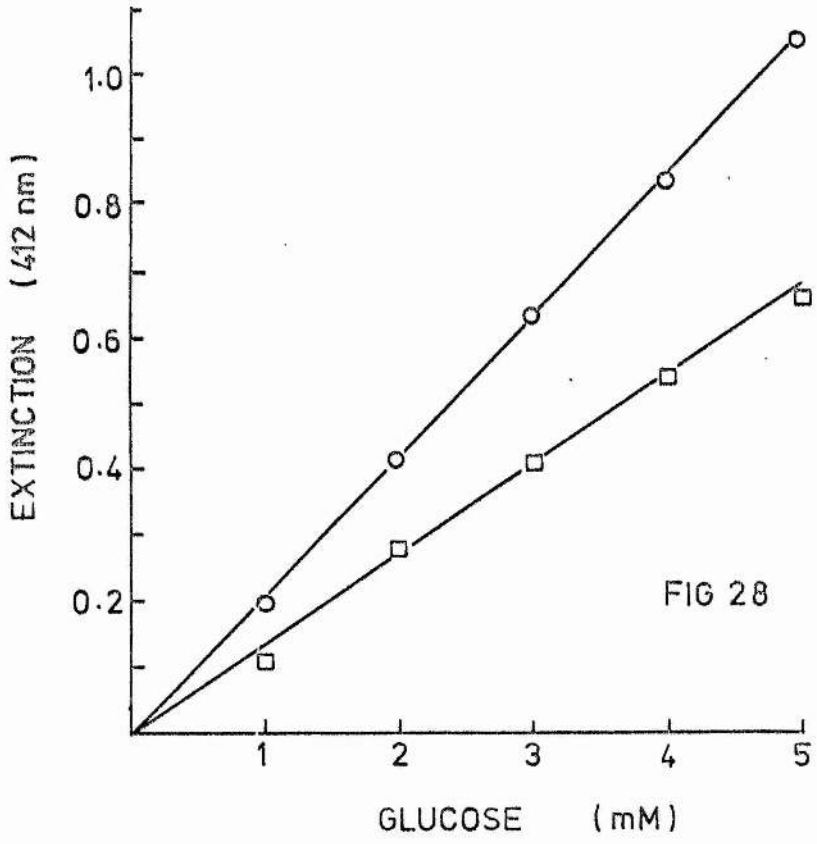

EXTINCTION (412 nm)
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
GLUCOSE (mM)

FIG 28

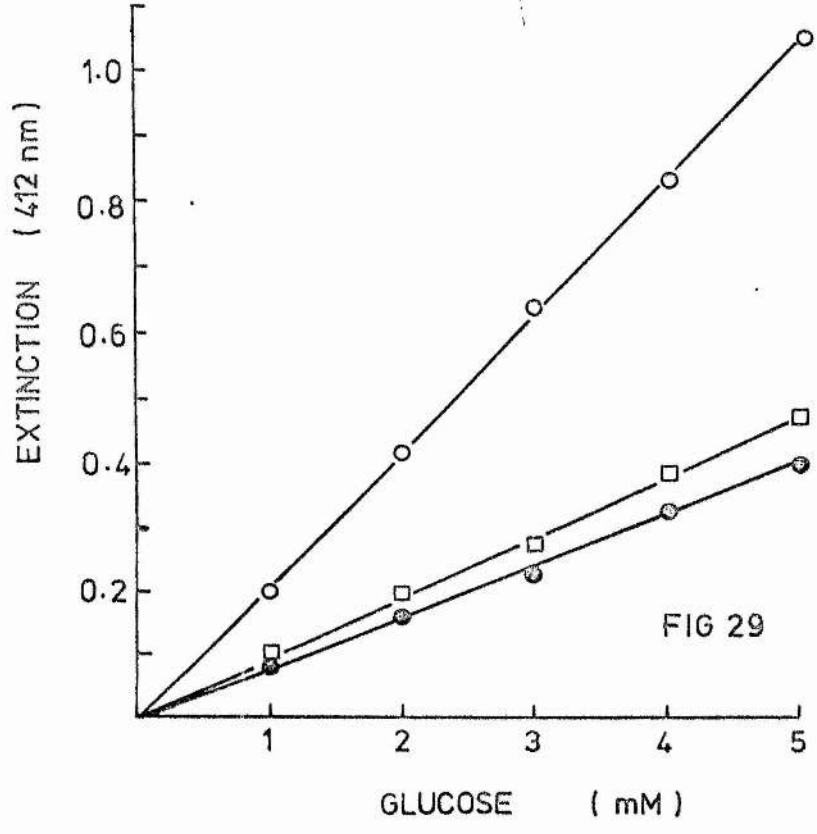

EXTINCTION (412 nm)
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
GLUCOSE (mM)

FIG 29

glucose was investigated in the following manner. Two 3 m lengths of HMDA-substituted GOD/CAT tubes were prepared, one using DMS as the alkylation reagent and the other using TOTFB. The enzyme coupling solution comprised 2 mg ml^{-1} glucose oxidase and 2 mg ml^{-1} catalase in 0.2 M-phosphate, pH 7.8. The combined activity of both enzymes co-immobilised to each derivative was measured by the analysis of 1-5 mM-glucose samples as described in section 3.6.5. The standard curves compiled for each derivative are shown in Fig 28. Both derivatives exhibited a linear response of extinction with glucose concentration in the range of samples examined. Although each derivative was capable of assaying samples as low as 0.5 mM-glucose, the TOTFB-alkylated GOD/CAT tube had 30% higher activity than the corresponding derivative prepared by DMS-alkylation. This result indicates the superiority of the former derivative for the analysis of glucose.

In most linked reactions involving two or more enzymes, there exists an optimum in the ratio of enzyme concentrations present that will produce the highest combined activity. In the case of co-immobilised enzymes, the product of one immobilised enzyme is the substrate for another, and therefore difficulties can be encountered in the resolution of the component activities. In the case of GOD/CAT tubes, the individual and combined activities of each immobilised enzyme were measured by means of three recirculation assays :
1) The immobilised glucose oxidase activity was measured by following the rate of glucose depletion as described in section 3.5.6 2) The catalatic activity of immobilised catalase in the presence of only 1.5 mM-H_2O_2 was measured by determining the K_{app} as described in section 3.5.4 3) The combined activity of both co-immobilised enzymes in the presence of methanol and glucose was measured by

Amount of GOD:CAT proffered in coupling solution (mg)	3:2	2:2	2:3	2:4	1.5:4	1:3	1:6
Units of glucose oxidase in coupling solution	630	420	420	420	315	210	210
Units of catalase in coupling solution x 10^3	30	30	45	60	60	45	90
Catalatic activity x 10^{-3} (cm^2 min^{-1})	11	-	12	14	14	-	15
Glucose oxidase activity (umol min^{-1} m^{-1})	1.81	1.80	1.80	1.79	1.8	1.25	0.80
Combined activity x 10^{-1} (umol HCHO min^{-1} m^{-1})	4.2	3.3	5.6	5.7	5.2	4.9	4.0
Extinction (412 nm) obtained from analysis of 4 mM-glucose	0.80	0.81	0.85	0.86	0.76	0.72	0.69

Table 7. The effect of different coupling solutions on the individual and combined activities displayed by GOD/CAT derivatives. 3 m lengths of glutaraldehyde activated/HMDA-substituted GOD/CAT derivatives were prepared under identical conditions as described in text. Each derivative was assayed for individual and combined activities as described in text.

following the rate of formaldehyde production as described in section 3.5.7. The combined activity was also measured by the insertion of each derivative into the flow system described in Fig 8c and analysing 4 mM-glucose samples in the manner described in section 3.6.5.

In an attempt to increase the combined activity of the co-immobilised enzymes on DMS-alkylated GOD/CAT tubes, the ratio of glucose oxidase : catalase in the coupling solutions was varied in the following manner. Seven 3 m lengths of activated/ IMDA-substituted nylon-tube were prepared as described in section 3.2. Co-immobilised enzyme derivatives were then prepared by filling these tubes with coupling solutions comprising : 630 and 30 000 ; 420 and 30 000 ; 420 and 45 000 ; 420 and 60 000 ; 315 and 60 000 ; 210 and 45 000 ; and 210 and 90 000 units of glucose oxidase and catalase respectively. In each case the units of glucose oxidase activity and catalase activity are expressed as described in sections 3.4.2 and 3.4.1. The individual and combined activities of the co-immobilised enzymes were measured as described above and the results are summarised in Table 7.

The immobilised glucose oxidase activity prepared from coupling solutions containing between 630 U glucose oxidase (30 000 U catalase) and 315 U glucose oxidase (60 000 U catalase) remained constant at 1.8 μmol min^{-1} m^{-1}. An increase in the catalase content of the coupling solution to 90 000 U catalase (210 U glucose oxidase) resulted in the immobilised glucose oxidase activity decreasing to 0.8 μmol min^{-1} m^{-1}. The immobilised catalatic activity increased from 11 x 10^{-3} to 15 x 10^{-3} cm^2 min^{-1} when the catalase content of the coupling solution was increased from 30 000 U catalase (630 U glucose oxidase) to 90 000 U catalase (210 U glucose oxidase). The combined activity of the co-immobilised derivative, as measured by the re-

circulation assay, increased from 4.2×10^{-1} to 5.7×10^{-1} umol min^{-1} m^{-1}, when the glucose oxidase concentration in the coupling solution decreased from 630 U (30 000 U catalase) to 420 U (60 000 U catalase). Increasing the catalase content of the coupling solution to 90 000 U catalase (210 U glucose oxidase) resulted in a decrease in the combined activity. Thus maximal combined activity was obtained with a derivative prepared from a coupling solution comprising 420 U glucose oxidase and 60 000 U catalase. This corresponded to a coupling solution containing 1 mg ml^{-1} glucose oxidase and 2 mg ml^{-1} catalase.

The observation that increasing the catalase content of the coupling solution from 60 000 U catalase (420 U glucose oxidase) to 90 000 U catalase (210 U glucose oxidase) resulted in a higher immobilised catalatic activity at the expense of both immobilised glucose oxidase activities and combined activities is in keeping with a situation where the catalase is immobilised in preference to the glucose oxidase owing to the high concentration of the former enzyme. This would also cause the combined activity to decrease if the glucose oxidase step was rate limiting.

Although a 7% increase in the combined activity of DMS-alkylated GOD/CAT tube was realised by adjustment of the enzymic composition of the coupling solution, the activity of such a derivative remained 23% lower than the corresponding derivative prepared by TOTFB-alkylation of the nylon-tube. Thus the alkylation of nylon-tube with TOTFB in place of DMS in the preparation of GOD/CAT tubes results in a more sensitive autoanalytical system for the determination of glucose.

The effect of spacers on the activity of TOTFB-alkylated GOD/CAT tubes was determined in the following manner. 3 m lengths of adipic acid dihydrazide -, succinic and dihydrazide- and HMDA-substituted GOD/CAT tubes were prepared as described in section 3.2. In each case the enzyme

coupling solution contained 1.5 mg ml^{-1} glucose oxidase and 2.0 mg ml^{-1} catalase in 0.2 M-phosphate, pH 7.8. Each was inserted into the flow system described in Fig 8c and the combined activity measured by the compilation of a glucose standard curve as described above. The results presented in Fig 29, show that the the adipic acid dihydrazide- and succinic acid dihydrazide- substituted derivatives displayed only 46% and 38% respectively of the combined activity of a HMDA-substituted GOD/CAT tube. Therefore in all subsequent studies on systems for the analysis of glucose, HMDA-substituted derivatives were prepared.

5.1.2. Operational Conditions

The sensitivity of many assay systems can be varied by changing the operational conditions. In particular, assay systems incorporating enzymes are often susceptible to pH variation owing to the pH dependence of enzymes. Therefore the effect of pH upon the activity of GOD/CAT tubes was studied in order to determine both the susceptibility of the assay system to fluctuations of pH and the optimal operational pH.

In order to determine the effect of pH upon the combined activity of the GOD/CAT system, pH profiles were first compiled for both soluble glucose oxidase and soluble catalase. The pH dependence of the former enzyme was studied by measuring the rate of oxygen depletion at different pH values in the manner described in section 3.4.2. The pH dependence of soluble catalase was studied by measuring the rate of H_2O_2 utilisation at different pH values as described in section 3.4.1. The results of these experiments are shown in Fig 30a and indicate that the glucose oxidase exhibits maximal activity in the range pH 5.5 - 5.7 and the catalase displays its maximal activity in the range pH 6.8. - 7.

Fig 30(a) shows the effect of pH upon the activity of :-

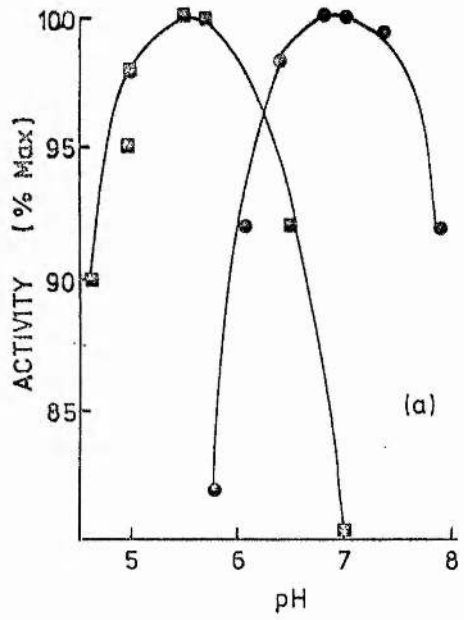
● soluble catalase ; and ■ soluble glucose oxidase.

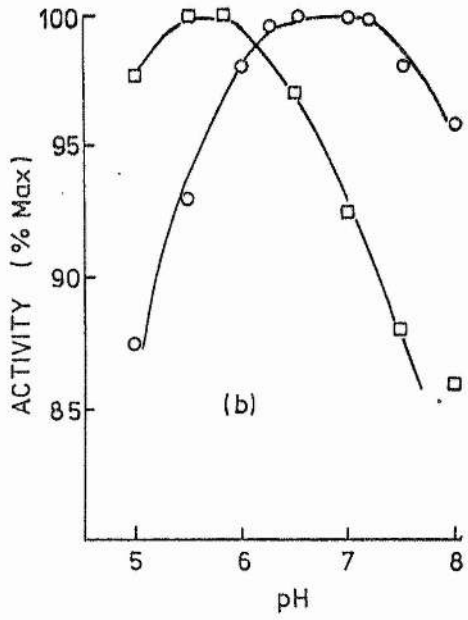
Fig 30(b) shows the effect of pH upon the activity of :-

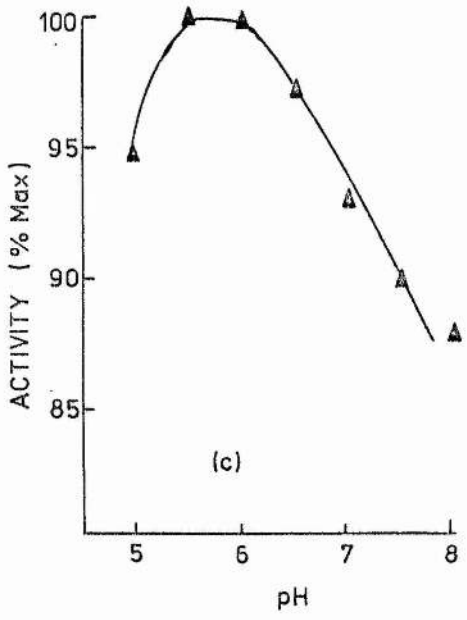
○ nylon-tube immobilised catalase ; and □ nylon-tube immobilised glucose oxidase.

Fig 30(c) shows the effect of pH upon the combined activity of a GOD/CAT derivative.

In all cases either 0.05 M- acetate or phosphate buffers were used


100
95
90
85
ACTIVITY (% Max)
(a)
5
6
7
8
pH


100
95
90
85
ACTIVITY (% Max)
(b)
5
6
7
8
pH


100
95
90
85
ACTIVITY (% Max)
(c)
5
6
7
8
pH

This indicates that the pH optimum of a linked reaction involving the soluble enzymes will lie in the range pH 5.5 - 7.0.

However, the immobilisation of some enzymes to nylon-tube has been shown to cause deviations in the pH profile (61). In order to ascertain whether any such effects occur with glucose oxidase and catalase, the effect of pH upon each of the immobilised enzymes was investigated in the following manner. A 1 m length of HMDA-substituted catalase tube and a 1 m length of HMDA-substituted glucose oxidase tube were prepared as described in section 3.2. The dependence of the immobilised catalase was studied by measuring the depletion of H_2O_2 occurring in a single pass of 1.5 mM-H_2O_2 through a 10 cm length of derivative as described in section 3.5.2. The pH dependence of a glucose oxidase tube was studied by the insertion of a 50 cm length of the derivative into the flow system described in Fig 7 and sampling 4 mM-glucose solutions as described in section 3.6.2. The results of these experiments are shown in Fig 30b and indicate that the immobilised glucose oxidase derivative exhibited maximal activity between a range pH 5.5 - 5.9 and the catalase tube displayed its maximal activity in the range pH 6.4 - 7.2. This indicates that the pH optimum of the linked reaction should lie in the range pH 5.5 - 7.2.

Comparison of the effect of pH upon the activities of soluble and immobilised glucose oxidase shows that deviations to occur in the pH profile of the immobilised enzyme, the general effect of which is that the latter is less dependent upon pH than the soluble enzyme. Similar results can be observed by comparison of the effect of pH on the activities of soluble and immobilised catalase. However, the pH profiles obtained for both soluble and insoluble catalase have to be treated with caution. Chance (62) has shown that the apparent pH profile obtained for the soluble enzyme is not a true reflection of the pH

dependence of the first order rate constant. This has been found to be independent of pH in the range 4 - 11, the spurious pH profile being due to the pH dependence of the rate of formation of the 'so-called' compound 2. The rate of formation of this inactive H_2O_2 - catalase complex is slowest between pH 6.8 and 7.0, resulting in the apparent pH optimum obtained at these values. It is probable that this effect also occurs in the immobilised enzyme although no experiments have been performed to verify the pH independence of the first order rate constant of the immobilised enzyme. The formation of compound 2 has been shown to inactivate the soluble enzyme (52). Therefore the pH profile of immobilised catalase, by indicating the pH range between which compound 2 formation is slowest, may also indicate the pH range of greatest stability to H_2O_2 exposure.

The effect of pH upon the combined activity of a co-immobilised GOD/CAT tube was further studied in the following manner. A 1 m length of HMDA-substituted- GOD/CAT tube was prepared as described previously. A 50 cm length of this derivative was assayed at different pH values by its insertion into the flow system described in Fig 8a, and the analysis of 4 mM-glucose samples as described in section 3.6.3. The results of this experiment are shown in Fig 30c and indicate that the combined activity has a pH optimum in the range pH 5.5 - 5.9. However only a 5% decrease in activity was observed over the range pH 5.0 - 7.0. This indicates the relative insensitivity to pH of a glucose analysis system using a co-immobilised GOD/CAT tube. The response of the combined activity of the co-immobilised enzymes is very similar to that of the immobilised glucose oxidase. As the pH dependence of most linked systems will be governed by the rate limiting step, these results suggest that in the case of the GOD/CAT tube assayed, glucose oxidase is the limiting step.

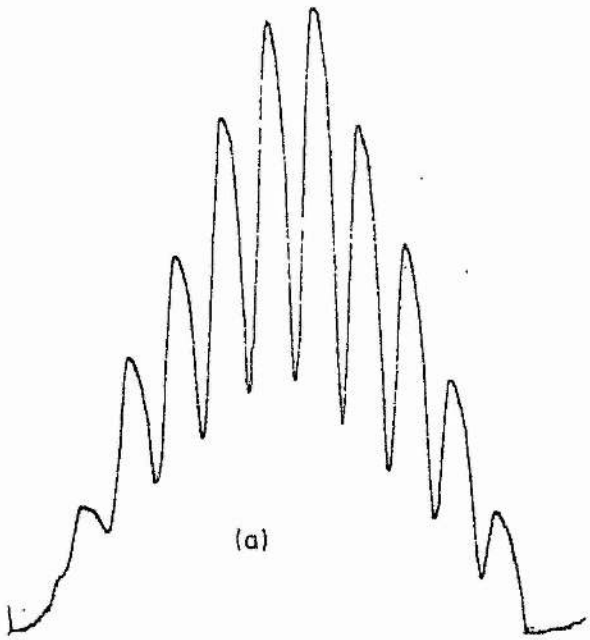
For the determination of glucose these results suggest that the highest combined activity of the two enzymes co-immobilised will be attained between pH 5.5 - 5.9. However, when describing the operational pH of any analyser system, effects such as enzymic stability, carry over etc, cannot be ignored. Operation at pH 6.9 sacrifices only 6% of the combined activity and should ensure increased stability of immobilised catalase due to the decreased rate of formation of compound 2. For these reasons the analysis of glucose was performed at pH 6.9.

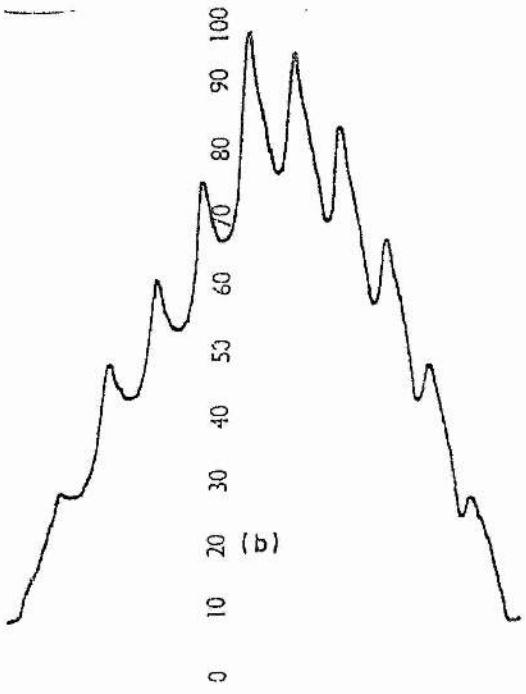
5.1.3. Operational Performance:

The operational performance of any autoanalytical system is an important factor deciding whether or not the system is acceptable in clinical laboratories for routine analytical purposes. The operational performance of GOD/CAT tubes in the autoanalysis of glucose was invostigated by studying the maximum assay capacity of the system, the extent of carry over associated with the system, and the maximum operating rate of the system.

The number of routine assays performed every day in a clinical laboratory requires that AA 1 autoanalyser systems are operated at sampling rates of 60 per h or higher. The maximum sampling rate that can be achieved for any system depends in part upon the extent of carry over. Carry over may be operationally defined as the cross-contamination of samples in the liquid stream of the autoanalyser. In principle, the sample pulse should exist in discrete sections of the air-segmented stream, resulting in a square-shaped peak on the recorder after the analytical reaction has taken place. However, in practise this is rarely achieved owing to the occurrence of such factors as sample adsorption onto the transmission tubing, and

Fig 31(a) and 31(b) . Recorder traces obtained from the determination of 2-10 mM-glucose samples using a DMS-alkylated GOD/CAT derivative and sampling rates of 30 and 60 per hour respectively.

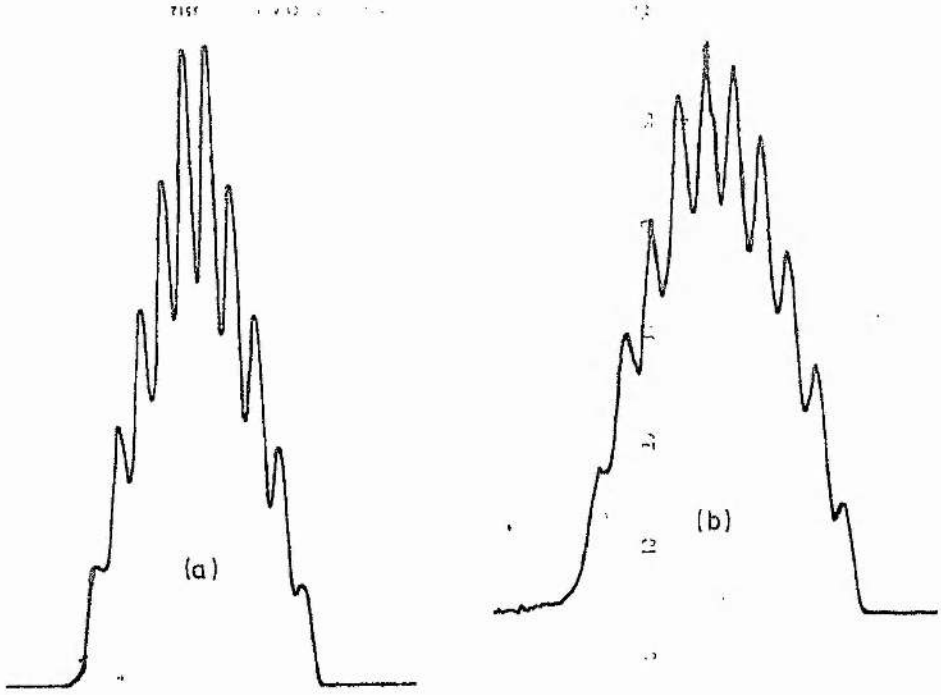

(a)


0
10
20
30
40
50
60
70
80
90
100
(b)

diffusional effects in the dialyser module. In the particular case of the systems incorporating nylon-tube immobilised enzymes, there is an extra consideration in that the substrates and/or reaction products may adsorb onto the surface of the nylon-tube. These factors introduce carry over into the system, resulting in a broadening of the peaks, usually accompanied by a corresponding decrease in peak height, and elevation of the base-line between the peaks. At high sampling rates only a small volume of air-segmented liquid separates each sample and therefore any change in peak shape caused by carry over may lead to extensive cross-examination of samples, resulting in poor accuracy and precision.

The extent of carry over associated with the analysis of glucose using TOTFB- and DMS-alkylated / HMDA-substituted GOD/CAT tubes was determined in the following manner. Duplicate samples of glucose were assayed by each derivative in the manner described in section 3.6.5 at sampling rates up to 60 per h. The recorder traces obtained from analysis of glucose by a DMS-alkylated derivative at sampling rates of 30 per h and 60 per h are shown in Figs 31a and 31b, and indicate that while errors of approximately 6% occurred at a sampling rate of 30 per h, errors of up to 12% occurred at 60 samples per h. Therefore higher sampling rates lead to unacceptable errors due to carry over. Similarly, the maximum rate attainable for the accurate analysis of formaldehyde standards was also 30 per h. Replacement of the enzyme tube with untreated nylon-tube enabled formaldehyde standards to be accurately analysed at rates up to 60 per h. These results suggest that DMS-alkylation of nylon-tubes promotes the adsorption of formaldehyde to the support. No increase in sampling rate was achieved by the addition of 0.2 M-NaCl and 1% (w/v) Brij 35

Fig 32(a) and 32(b) . Recorder traces obtained from the determination of 2-10 mM-glucose samples at a sampling rate of 60 per hour using : (a) A GOD/CAT derivative that had been prepared by means of reaction with TOTFB for 10 min ;
(b) A GOD/CAT derivative that had been prepared by means of reaction with TOTFB for 30 min.


(a)
(b)

detergent to the reagents. This implies the presence of strong adsorption of substrates and/or reaction products to the nylon-tube.

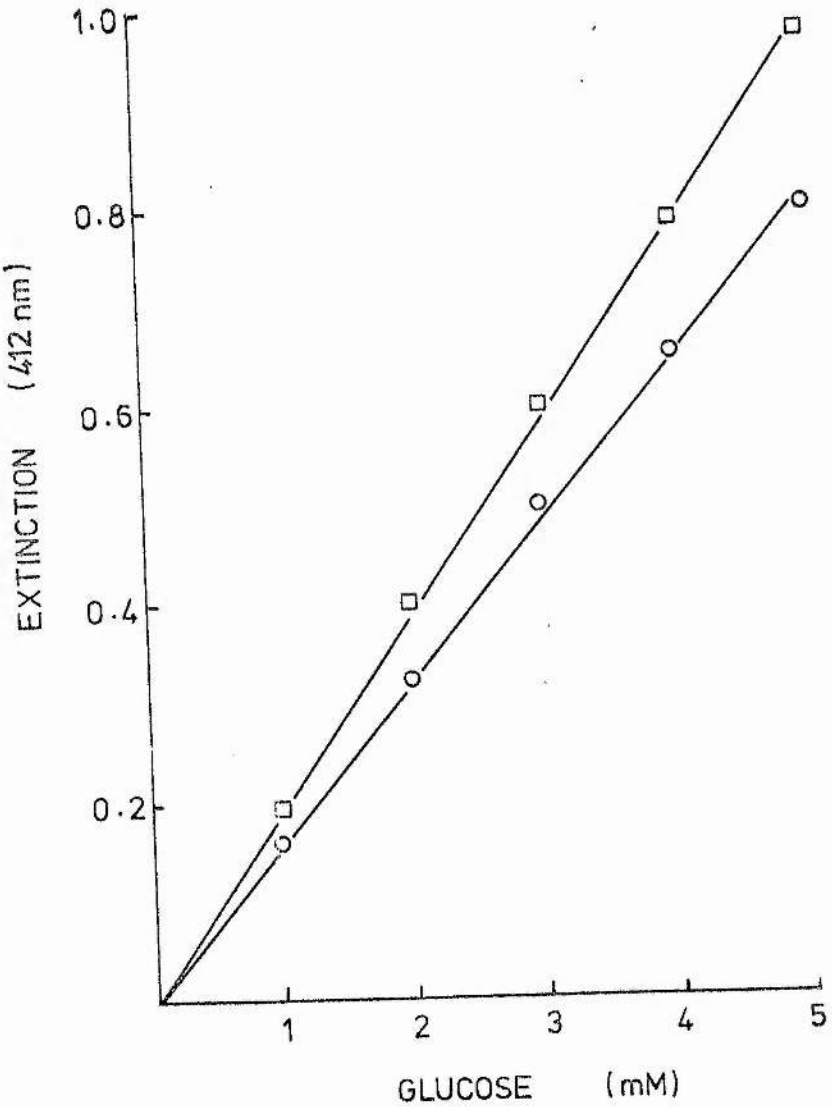
A TOTFB-alkylated GOD/CAT tube was examined in a similar manner to that described above. The recorder trace obtained from the analysis of glucose at a sampling rate of 60 per h is shown in Fig 32a, and indicates that the required accuracy of glucose determination can be attained by sample rates as high as 60 per h. This suggests that nylon treated with TOTFB maintains a greater surface integrity than DMS-alkylated nylon, thus limiting the extent of adsorption of substrates and/or reaction products onto the surface of the nylon-tube.

The effect of longer alkylation times on the carry over associated with TOTFB-alkylated tubes was further examined in the following manner. Three 3 m lengths of HMDA-substituted GOD/CAT derivatives were prepared under the conditions described in section 3.2 except that the alkylation reaction was allowed to continue for 10, 20, and 30 min respectively. The recorder traces obtained by analysis of glucose at sampling rates of 60 per h using tubes prepared by 10 min and 30 min alkylation times are shown in Figs 32a and 32b. These results indicate that only the derivative prepared by the 10 min alkylation time could be used to determine glucose at rates as high as 60 per h. This implies that prolonged alkylation of the nylon-tube increases the extent of adsorption of substrates and/or reaction products onto the surface of the nylon. This effect is manifested by increased carry over in the system thus neceessitating lower sampling rates.

The inability of DMS-alkylated GOD/CAT tubes to analyse glucose samples at rates exceeding 30 per h greatly decreases the utility of such a derivative for autoanalytical purpose. As previously stated,

Fig 33 . The effect of 10 000 aqueous glucose assays upon the activity of a GOD/CAT derivative.

□ Activity immediately after preparation ; O Activity after 10 000 assays.


1.0
0.8
0.6
0.4
0.2
EXTINCTION (412 nm)
1
2
3
4
5
GLUCOSE (mM)

the number of glucose analyses performed in clinical laboratories necessitates the use of systems capable of performing at least 60 assays per h. Existing methods for glucose determination (both chemical and enzymic) meet this requirement, and therefore these rates have to be achieved in immobilised enzyme systems if the latter systems are to be of any significance. The DMS-alkylated GOD/CAT tube assay system has been shown to exhibit less sensitivity in glucose analysis and less operational capacity than the corresponding TOTFB-alkylated derivative. For these reasons, DMS-alkylated GOD/CAT tubes were deemed unsuitable for the auto-analysis of glucose.

The applicability of a nylon-tube immobilised enzyme system for the determination of metabolites such as glucose is also dependent upon the stability of the immobilised preparation. For reasons of economics and convenience, a nylon-tube immobilised enzyme assay system has to be capable of performing large numbers of assays before it can be accepted as a suitable analytical tool. The operational stability of GOD/CAT tubes in the automated analysis of aqueous glucose solutions was determined in the following manner. A 3 m length of HMDA-substituted GOD/CAT tube was prepared as described in section 3.2. The enzyme coupling solution comprised 2 mg ml^{-1} glucose oxidase and 2 mg ml^{-1} catalase in 0.2 M-phosphate pH 7.8. The derivative was inserted into the flow system described in Fig 8c and 1-5 mM-glucose samples were assayed at a rate of 60 per h. At intervals the sampling rate was decreased to 40 per h and standard curves were compiled. In this way the combined activity of the co-immobilised derivative was monitored throughout a period of one week during which time 10 000 continuous assays were performed. The standard curves compiled before and after the continuous assay of 10 000 glucose samples are shown in Fig 33. Although a 16% decrease

Fig 34 . Standard curve for the estimation of glucose using a GOD/CAT derivative inserted into a flow system containing a dialyser module.

□ Sampling rate of 40 per h

○ Sampling rate of 60 per h

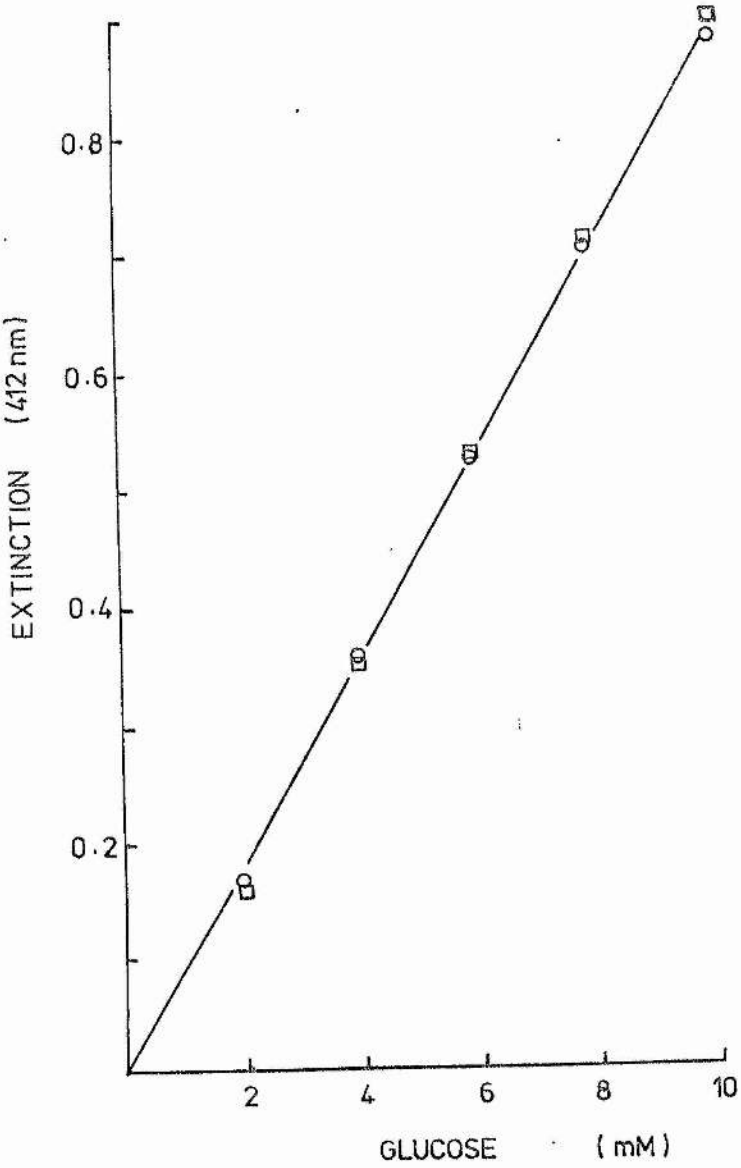
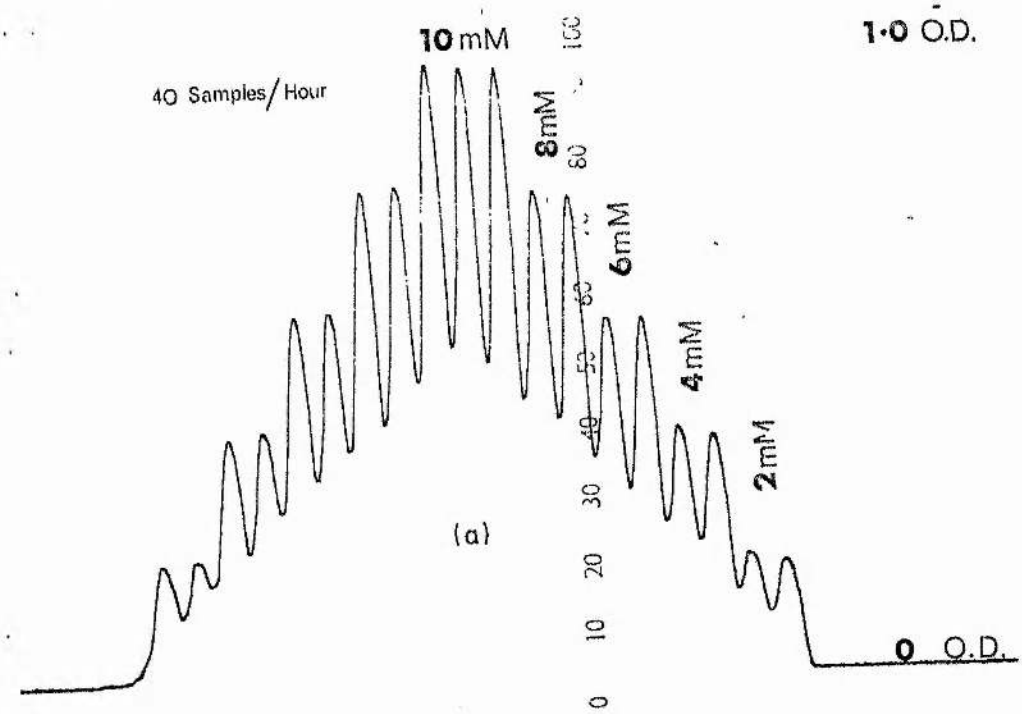
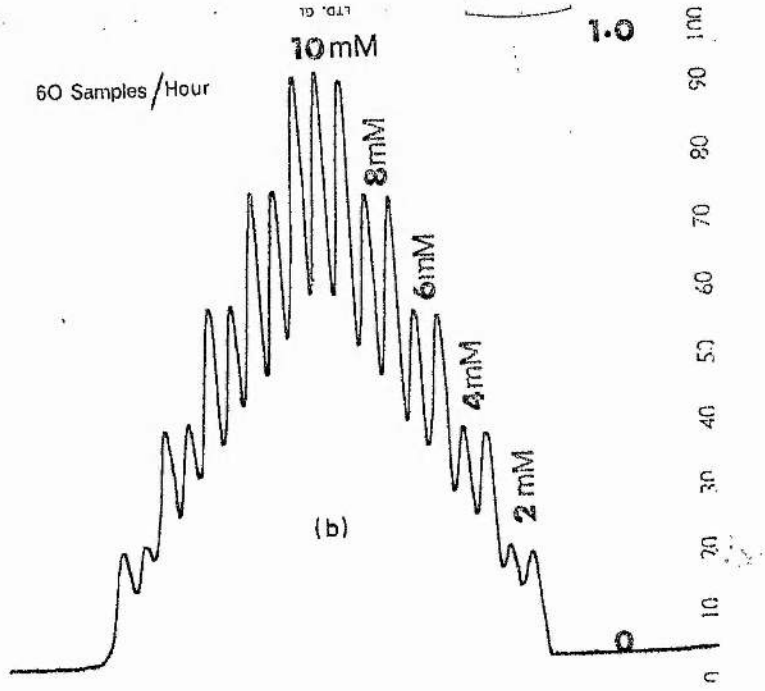

EXTINCTION (412 nm)
0·8
0·6
0·4
0·2
2
4
6
8
10
GLUCOSE (mM)

Fig 35(a) and 35(b) . Recorder traces obtained from the determination of 2-10 mM-glucose samples using a GOD/CAT derivative inserted into a flow system containing a dialyser module. Samples were determined at rates of 40 and 60 per h respectively.


10 mM
1·0 O.D.
40 Samples/Hour
8mM
6mM
4mM
2mM
(a)
0 O.D.
0
10
20
30
40
50
60
80
100


10 mM
1·0
60 Samples/Hour
8mM
6mM
4mM
2 mM
(b)
0
10
20
30
40
50
60
70
80
90
100

in activity occurred during this period, adequate combined activity remained to ensure the sensitivity of the assay. Thus one GOD/CAT tube is capable of performing at least 10 000 assays indicating the utility of such a system for the analysis of glucose.

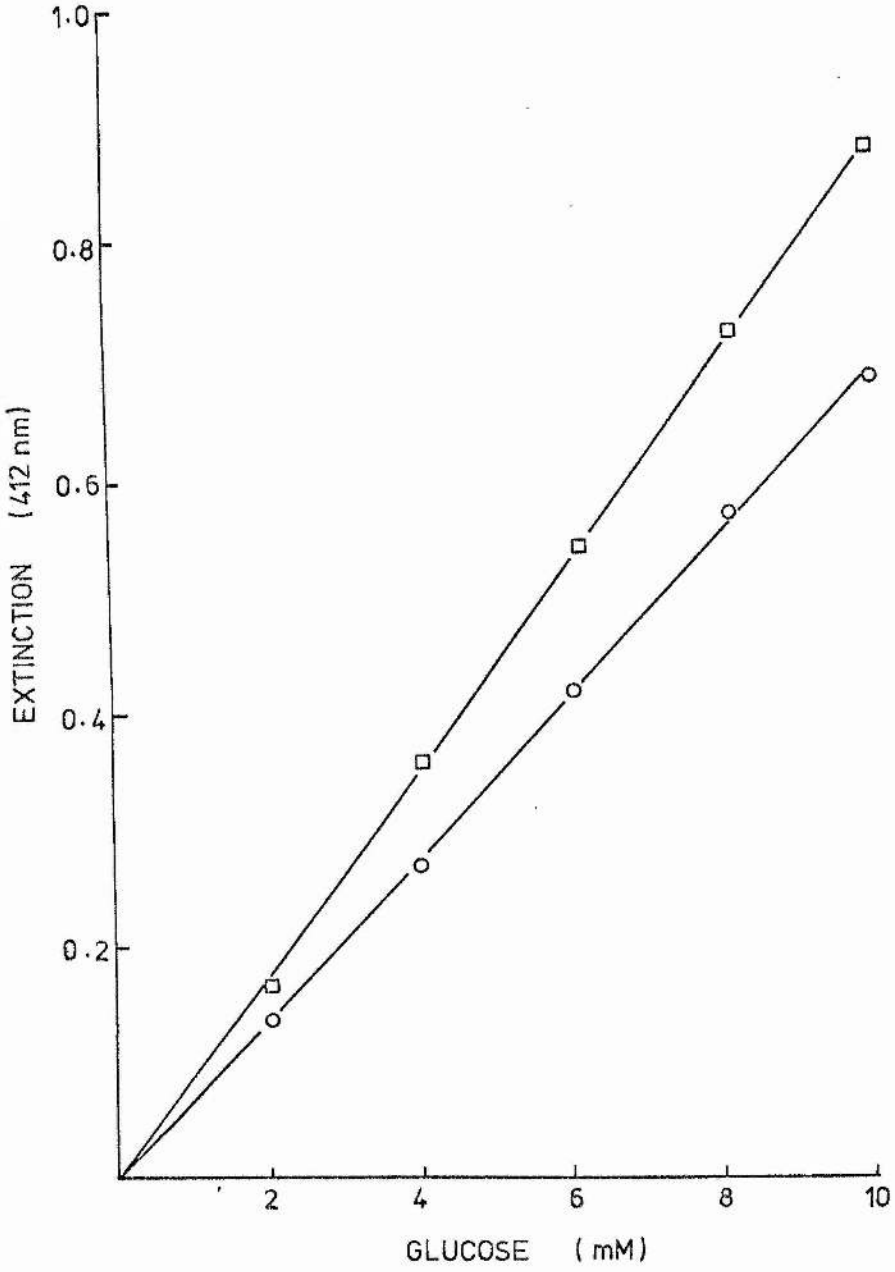
5.1.4. Determination of Serum Glucose

The experiments performed in the previous sections to determine the utility of a GOD/CAT system for glucose determinations have involved the analysis of aqueous glucose solutions. However, most clinical analyses are performed upon whole-blood (9). For this reason all systems involving enzyme reactions have to include dialysers in order to prevent interference of the assay by blood enzymes. This is achieved by dialysis of the sample whereby the low molecular weight metabolities, present in the serum, are collected into an air-segmented recipient stream. In the case of glucose determination using GOD/CAT derivatives, a dialyser has to be incorporated into the system to prevent possible interference by proteins present in blood on the assay system. The flow system used for the determination of glucose in serum is shown in Fig 10a. The normal concentration of glucose in venous blood lies in the range 3.6 - 6.3 mM-glucose (63), and any autoanalytical system for the clinical determination of glucose must display adequate sensitivity for samples in the range 0.5 mM-10 mM-glucose. The sensitivity of glucose determination obtained by this system and its operation rate were first determined by the analysis of 2 - 10 mM-glucose samples at sampling rates of 40 and 60 per h. The standard curves thus obtained are shown in Fig 34 and indicate that the system displays adequate sensitivity for use as a method for the determination of glucose. The recorder traces from which the standard curves

Fig 36 . The effect of 10 000 serum assays upon the activity of a GOD/CAT derivative.

□ Activity immediately after preparation ; O Activity after 10 000 serum assays.

of



were complied are shown in Figs 35a and 35b and indicate that no marked carry over occurs at sampling rates up to 60 per h.

The operational stability of GOD/CAT tubes to the conditions employed in the analysis of aqueous glucose has been previously discussed. However, serum is a complex solution in which enzyme inhibitors and deactivators may also be present. While the dialysis of serum removes compounds of high molecular weight from the assay system, low molecular weight compounds are collected in the air-segmented recipient stream. In order to determine whether such compounds have an adverse effect upon the combined activity of a GOD/CAT derivative it's stability to serum analysis was investigated in the following manner. A 3 m length of HMDA-substituted GOD/CAT tube was inserted into the flow system described in Fig 10a. 2 - 10 mM-glucose samples were assayed at a sampling rate of 40 per h, and a standard curve compiled as described in section 3.6.6. Bovine serum samples were then assayed continuously at sampling rates of 60 per h for a period of one week during which time 10 000 serum assays were performed. The stability of the derivative was determined by comparison with a standard curve compiled after the completion of the serum assays. The results of this experiment are shown in Fig 36 and indicate that although the activity of the derivative decreased by 22% over 10 000 assays, sufficient activity remained for the analysis of further samples. Thus the stability of a GOD/CAT tube is sufficient for the determination of at least 10 000 serum assays per derivative.

The application of co-immobilised GOD/CAT derivatives for the clinical determination of glucose has therefore been shown to attain the standards of sensitivity, speed of operation, and stability required for a clinical analytical assay system.

5.2. DETERMINATION OF GLUCOSE USING NYLON-TUBE IMMOBILISED GLUCOSE OXIDASE IN SERIES WITH NYLON-TUBE IMMOBILISED CATALASE

5.2.1. Operational Conditions and Performance

As discussed in the previous section, the peroxidatic utilisation of H_2O_2 is more efficient when the H_2O_2 is continuously generated *in situ* rather than added in discrete concentrations. However, results previously presented in section 4.4.3 show that a HMDA-substituted 2mm-bore nylon-tube immobilised catalase derivative prepared by TOTFB alkylation is capable of using 83% of a 4 mM-H_2O_2 sample in a peroxidatic manner, whereas a DMS-alkylated derivative could only use 37% of the H_2O_2 in such a manner. These results suggest the possibility of determining glucose by means of a system incorporating separate lengths of TOTFB-alkylated nylon-tube immobilised derivatives of glucose oxidase and catalase deployed in series. The combined activities of the sequential TOTFB-alkylated derivatives and the co-immobilised GOD/CAT tube were compared in the following manner. 2 m lengths of HMDA-substituted/1 and 2 mm-bore/nylon-tube immobilised glucose oxidase, 2 m lengths of HMDA-substituted/1 and 2 mm-bore/nylon-tube immobilised catalase and a 3 mm length of HMDA-substituted/1 mm bore/nylon-tube co-immobilised glucose oxidase and catalase were prepared as previously described. In each case the enzyme coupling solution comprised 3 mg ml^{-1} of the respective protein in 0.2 M-phosphate, pH 7.8. The overall activity of the co-immobilised derivative and both the 1 mm and 2 mm bore separately immobilised derivatives were measured by their insertion into the flow system described in Fig 10b and the compilation of standard curves for glucose as described in section 3.6.8. The results of these experiments are shown

Fig 37 . Comparison of the combined activity of a GOD/CAT derivative with that of nylon-tube immobilised derivatives of glucose oxidase and catalase placed in series.

○ 1 mm bore nylon-tube GOD/CAT

▩ 1 mm bore nylon-tube derivatives of glucose oxidase and catalase in series.

□ 2 mm bore nylon-tube derivatives of glucose oxidase and catalase in series.

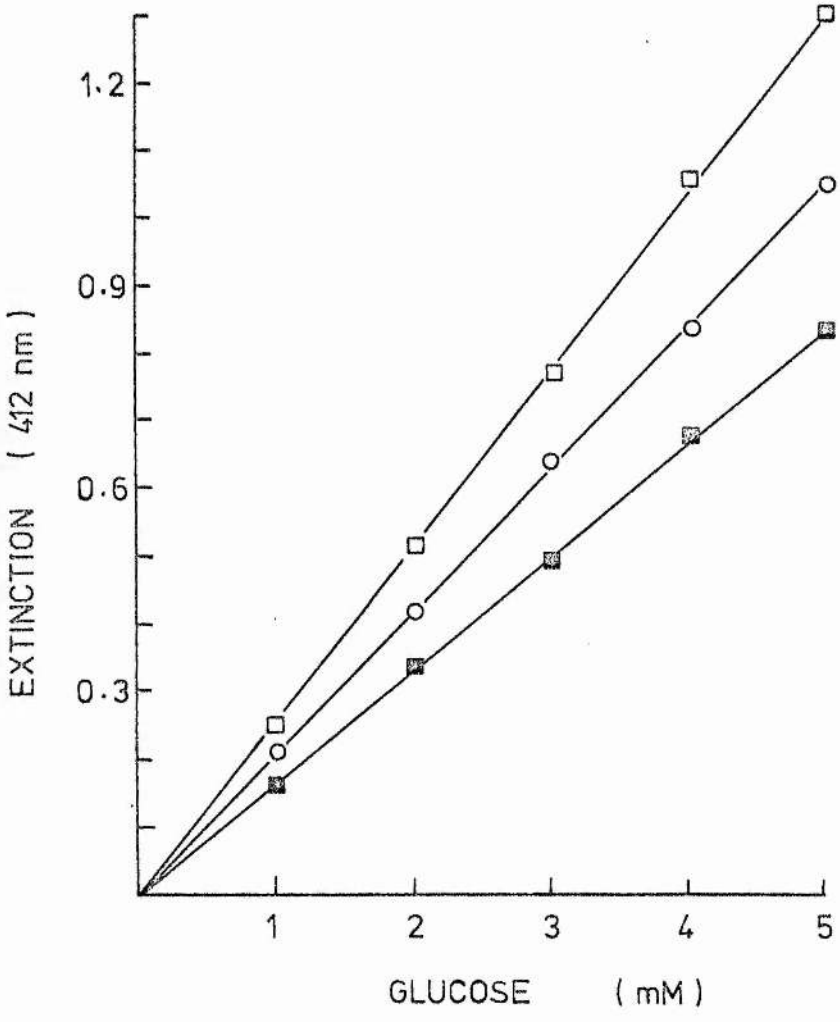
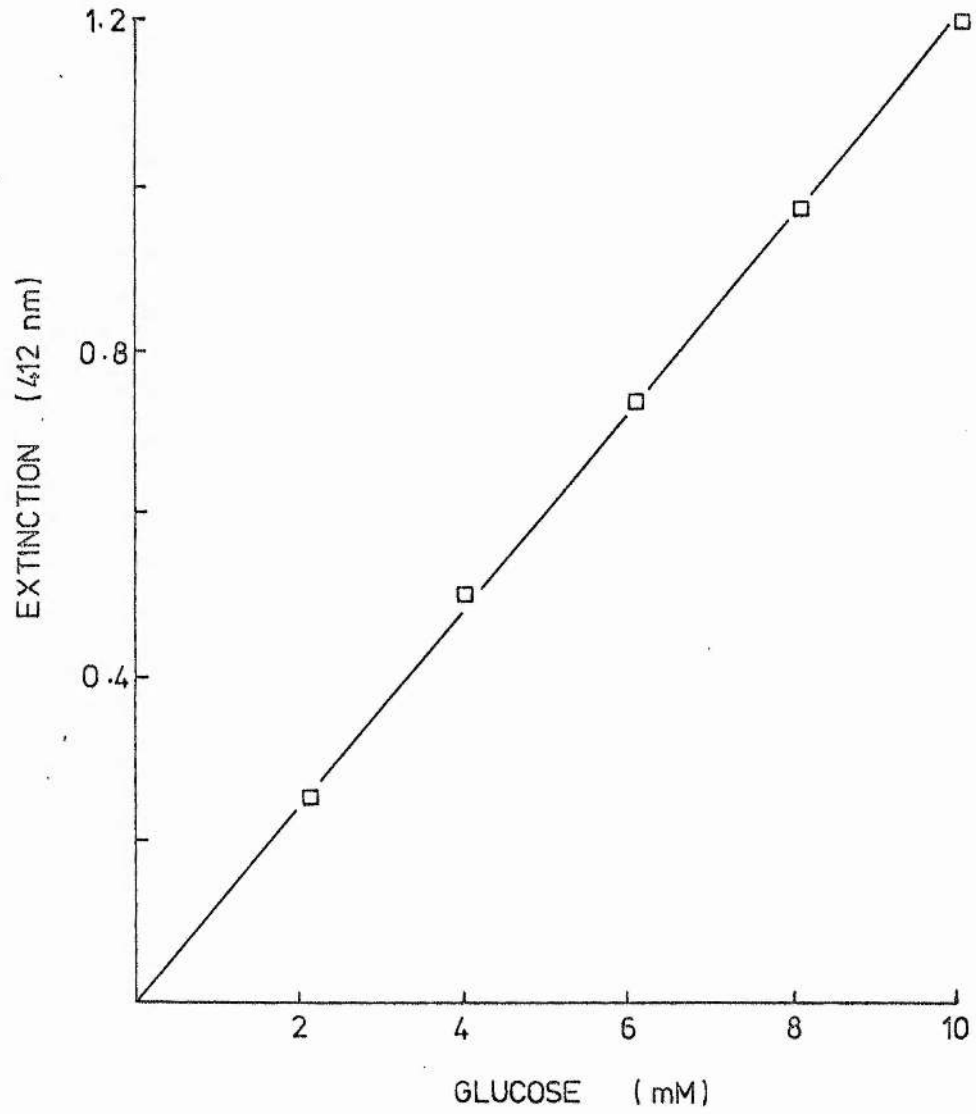

EXTINCTION (412 nm)
1.2
0.9
0.6
0.3
1
2
3
4
5
GLUCOSE (mM)

Fig 38 . Standard curve for the estimation of glucose using nylon-tube immobilised derivatives of glucose oxidase and catalase placed in series.


1.2
0.8
0.4
EXTINCTION (412 nm)
2
4
6
8
10
GLUCOSE (mM)

in Fig 37 and indicate that the combined activity of the two 1 mm bore separately immobilised derivatives was only 20% less than that of the 1 mm bore co-immobilised derivative. The combined activity of the two 2 mm bore enzyme tubes was 29% higher than the 1 mm-bore co-immobilised enzyme tube. This can be explained by the greater surface area of the former derivatives. It has already been shown that the activity of immobilised catalase is increased by use of tubes with larger diameter and this result suggests that a similar effect also occurs with glucose oxidase thus causing greater H_2O_2 production as well as greater peroxidatic utilisation of the H_2O_2 by catalase. Thus the use of 2 m lengths of 2 mm bore glucose oxidase tube and 2 mm bore catalase tube in the determination of glucose provide an analytical system exhibiting adequate sensitivity for use in clinical laboratories.

As discussed previously, it is necessary to include a dialyser into the flow system when assaying whole-blood by any method involving glucose oxidase and catalase. The system used for the determination of glucose in serum is shown in Fig 10b. The sensitivity of glucose determinations using assay this system was first determined by the compilation of a standard curve for glucose as described in section 3.6.7. The results are shown in Fig 38 and indicate that adequate sensitivity is achieved by this system.

The effect of the length of each derivative on the combined activity was determined in the following manner. In one experiment, the length of catalase tube was maintained at 2 m while the length of glucose oxidase tube was varied from 0.5 m to 2.0 m. In another experiment, the length of the glucose oxidase tube was kept constant at 2 m and the length of catalase tube varied from 0.25 m to 2 m. In each case the combined activity of the derivatives was

Fig 39 . Dependence of the combined activity of nylon-tube immobilised derivatives of glucose oxidase and catalase upon the length of glucose oxidase tube. In each case the length of the catalase tube was kept constant at 2 m .

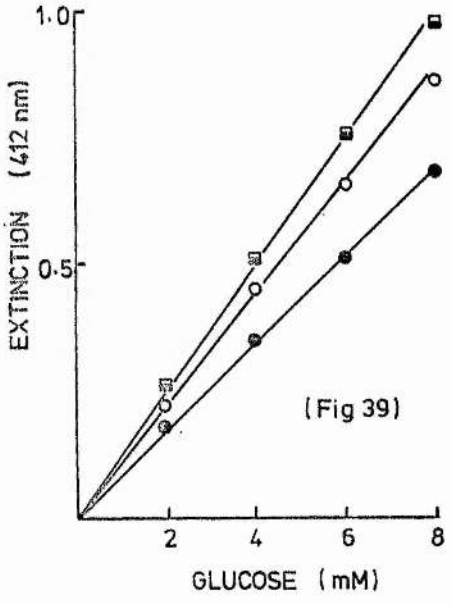
■ 2 m ; ■ 1.5 m ; ○ 1.0 m ; ● 0.5 m of glucose oxidase tube

Fig 40 . Dependence of the combined activity of nylon-tube immobilised derivatives of glucose oxidase and catalase upon the length of catalase tube. In each case the length of glucose oxidase tube was kept constant at 2 m.

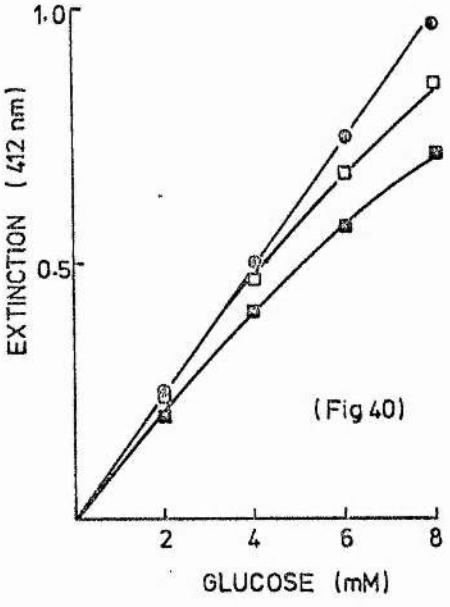
◐ 2 m ; ◐ 1.0 m ; □ 0.5 m ; ■ 0.25 m length of catalase tube.

Fig 41 . Dependence of peroxidatic activity upon the length of nylon-tube immobilised catalase.

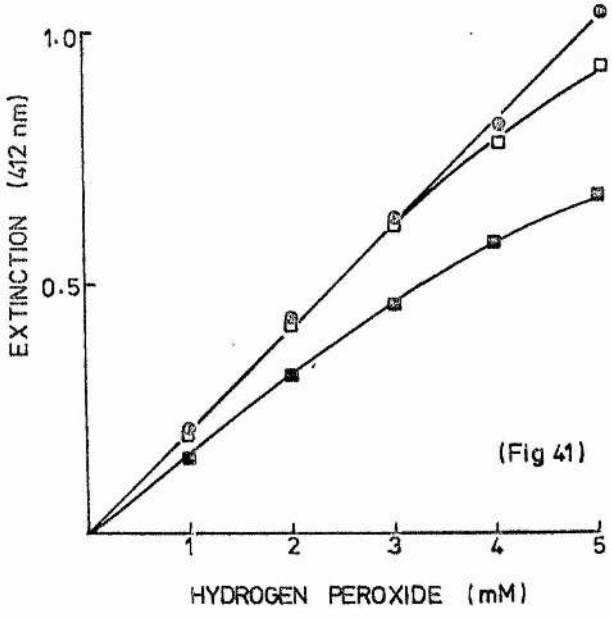
◐ 2.0 m ; ◐ 1.0 m ; □ 0.5 m ; ■ 0.25 m length of catalase tube



(Fig 39)



(Fig 40)



(Fig 41)

measured by their insertion into the flow system described in Fig 10b and the compilation of a standard curve as described previously. Where the catalase tube length was varied, a standard curve for H_2O_2 was also compiled in order to monitor the changing peroxidatic activity of the catalase derivative. The results of these experiments are shown in Figs 39-41. Decreasing the length of the glucose oxidase tube from 2 m to 1.5 m resulted in no loss in combined activity, whereas further decreases in tube length resulted in corresponding decreases in combined activity. This suggests that the use of 2 m and 1.5 m lengths of glucose oxidase ensures maximal conversion of glucose in the sample. This will not necessarily be 100% of the total glucose owing to the inability of glucose oxidase to utilise α - D-glucose.

Decreasing the length of the catalase tube employed in the assay system from 2 m to 1.0 m resulted in no decrease in the combined activity. This suggests that 1 m of derivative is required for maximal peroxidatic conversion of H_2O_2. Further decreases in the length of the catalase tube resulted in decreases in both combined and peroxidatic activity suggesting that the peroxidatic conversion of the H_2O_2 produced by the glucose oxidase reaction was less efficient at these lengths.

These experiments suggest that 1.5 m lengths of each derivative are adequate to ensure maximal sensibility of glucose analysis.

The operational stability of the system for the determination of serum glucose was investigated in the following manner. 1.5 m lengths of nylon-tube immobilised glucose oxidase and nylon-tube immobilised catalase were inserted into the flow system described in Fig 10b, and a standard curve for glucose compiled by the analysis of 2 - 10 mM-glucose samples. Serum samples were then assayed at a rate of 60 per h for a period of 50 h, after which a further standard curve was compiled.

No loss in combined activity was observed over 3 000 continuous assays on serum, showing that the stability of the assay system is adequate for clinical analytical purposes.

Two control serum samples of known glucose concentration were assayed using this system. The immobilised enzyme gave values of 5.24 mM- and 2.62 mM-glucose. These results compared with values of 5.33 mM- and 2.66 mM-glucose respectively obtained by analysis of the serum using the autoanalytical procedure of Trinder (7). Thus a correlation between the Trinder (soluble glucose oxidase/peroxidase) system and the immobilised glucose oxidase + catalase system was obtained. This indicates that the estimation of glucose using the immobilised enzyme system described in this section, produces results that correspond with those obtained by methods already in use in clinical laboratories.

5.3. DETERMINATION OF GLUCOSE USING NYLON-TUBE IMMOBILISED DERIVATIVES OF GLUCOSE OXIDASE, CATALASE, AND ALDEHYDE DEHYDROGENASE IN SERIES

There exists two schools of thought with regard to metabolite determination using autoanalytical systems incorporating enzymic reactions. One is that such systems should be brought down to an 'autoanalytical common denominator'. This common step is generally regarded as the production (or disappearance) of the coenzyme NADH which can be monitored spectrophotometrically at 340 nm. The other school of thought argues that autoanalysis using coenzymes is too expensive at the present time owing to the volume of reagents used and the cost of commercially available preparations. However, there now exist a new range of analysers in which the volumes of the reagent streams have been drastically reduced, thus decreasing the costs associated with coenzyme utilisation. Autoanalyser systems

involving coenzymes may therefore become more economically viable in the future.

This section indicates the method by which the detection of glucose using nylon-tube immobilised derivatives of glucose oxidase and catalase can be converted from spectrophotometric analysis of the Hantzsch reaction at 412 nm, to spectrophotometric analysis of NADH production at 340 nm. This system also involves the peroxidatic action of immobilised catalase. However, in this instance, ethanol is converted to acetaldehyde by catalase in the presence of the H_2O_2 generated by the primary glucose oxidase reaction. The acetaldehyde in turn is oxidised to acetate by aldehyde dehydrogenase with the concommitant reduction of NAD^+ to NADH. The latter is monitored spectrophotometrically at 340 nm, thus providing an assay protocol for glucose that can be measured at 340 nm.

5.3.1. Preparation of Nylon-Tube Immobilised Aldehyde Dehydrogenase

The effect of different methods of enzymic attachment on the retention of immobilised activity was studied in the following manner. The use of different bifunctional reagents were investigated by the preparation of 2 m lengths of diethyladipimidate- and glutaraldehyde-activated/HMDA-substituted/ 1 mm bore/nylon-tube immobilised aldehyde dehydrogenase. In each case the derivative was assayed for bound protein and enzyme activity by the methods described in sections 3.3 and 3.5.8. The results of these experiments are summarised in Table 8. The glutaraldehyde- and diethyladipimidate-activated derivatives bound 0.7 and 0.95 mg m^{-1} protein respectively. The derivatives displayed activities of 0.02 and 0.14 umol min^{-1} m^{-1} respectively, corresponding to protein specific activities of 0.4 and 2.2 μmol min^{-1} mg^{-1}. Thus use of diethyladipimidate in place of glutaraldehyde produced a 7-fold

more active immobilised derivative. The relative lack of specificity of glutaraldehyde compared with diethyladipimidate has already been discussed in section 4.1.5 with regard to the immobilisation of catalase and similar reasoning may be used in the case of aldehyde dehydrogenase.

	1Nt/HMDA/G/AD	1Nt/HMDA/A/AD	2Nt/HMDA/A/AD	2Nt/AH/A
Total protein bound (mg)	1.4	1.9	2.0	1.03
Protein bound per meter of tube (mg m^{-1})	0.70	0.95	1.0	0.52
Activity (umol min^{-1} m^{-1})	0.02	0.14	0.264	0.21
Specific activity (umol min^{-1} m^{-1} mg^{-1})	0.4	2.2	4.0	6.1

Table 8. Preparation of nylon-tube immobilised aldehyde dehydrogenase. Derivatives were prepared under identical conditions as described in text. In each case the enzyme coupling solutions comprised 2 mg ml^{-1} aldehyde dehydrogenase in 0.2 M-phosphate, pH 7.8. Derivatives were assayed for bound protein and enzymic activity as described in text.

The effect of the diameter of nylon-tube on the activity of immobilised derivatives of aldehyde dehydrogenase was also investigated. A 2 m length of diethyladipimidate-activated/HMDA-substituted/2 mm bore/ nylon-tube immobilised aldehyde dehydrogenase was prepared in the manner described in section 3.2. The derivative was assayed for bound protein and enzymic activity as described in sections 3.3 and 3.5.8. The results of this experiment are summarised in Table 8. The 2 mm bore aldehyde dehydrogenase tube bound 1.0 mg m^{-1} protein and had an activity of 0.264 umol min^{-1} m^{-1}, corresponding to a protein specific activity of 4.0 umol min^{-1} mg^{-1}. Thus use of the 2 mm bore tube in place of 1 mm

bore tube increased the activity of the immobilised derivative by 86%. A similar result was obtained for the immobilisation of catalase onto 2 mm bore nylon-tube and has been discussed in chapter 4.

The effect of different spacers on the retention of immobilised activity was investigated by the preparation of diethyladipimidate-activated/adipic acid dihydrazide-substituted/ 2 mm bore nylon-tube immobilised aldehyde dehydrogenase in the manner described in section 3.2. The derivative was assayed for bound protein and enzyme activity as described in sections 3.3 and 3.5.8. The results of this experment are also summarised in Table 8. The adipic acid dihydrazide-substituted derivative bound only 52% as much protein as the corresponding HMDA-substituted tube, but exhibited 78% of the activity associated with the latter derivative.

Collectively from these results it was deemed necessary to employ diethyladipimidate-activated, 2 mm bore substituted nylon-tube immobilised derivatives of aldehyde dehydrogenase in an autoanalytical assay of glucose.

5.3.2. The Autoanalytical Determination of Glucose and Stability to Serum

The flow system employed for the determination of glucose using nylon-tube immobilised derivatives of glucose oxidase, catalase and aldehyde dehydrogenase is shown in Fig 13. The sensitivity of glucose determination using this assay protocol was first investigated using 2 m lengths of glutaraldehyde-activated/HMDA-substituted/2 mm bore nylon-tube immobilised derivatives of glucose oxidase and catalase in series with 1) a 2 m length of diethyladipimidate-activated/HMDA-substituted/2 mm bore aldehyde dehydrogenase tube ; and 2) a 2 m length of diethyladipimidate-activated/adipic acid dihydrazide substituted/2 mm

Fig 42 . Standard curves for the estimation of glucose using nylon-tube immobilised derivatives of glucose oxidase , catalase and aldehyde dehydrogenase in series.

In each case identical derivatives of glucose oxidase and catalase were used

□ HMDA-substituted nylon-tube immobilised aldehyde dehydrogenase

O Adipic acid dihydrazide-substituted nylon-tube immobilised aldehyde dehydrogenase

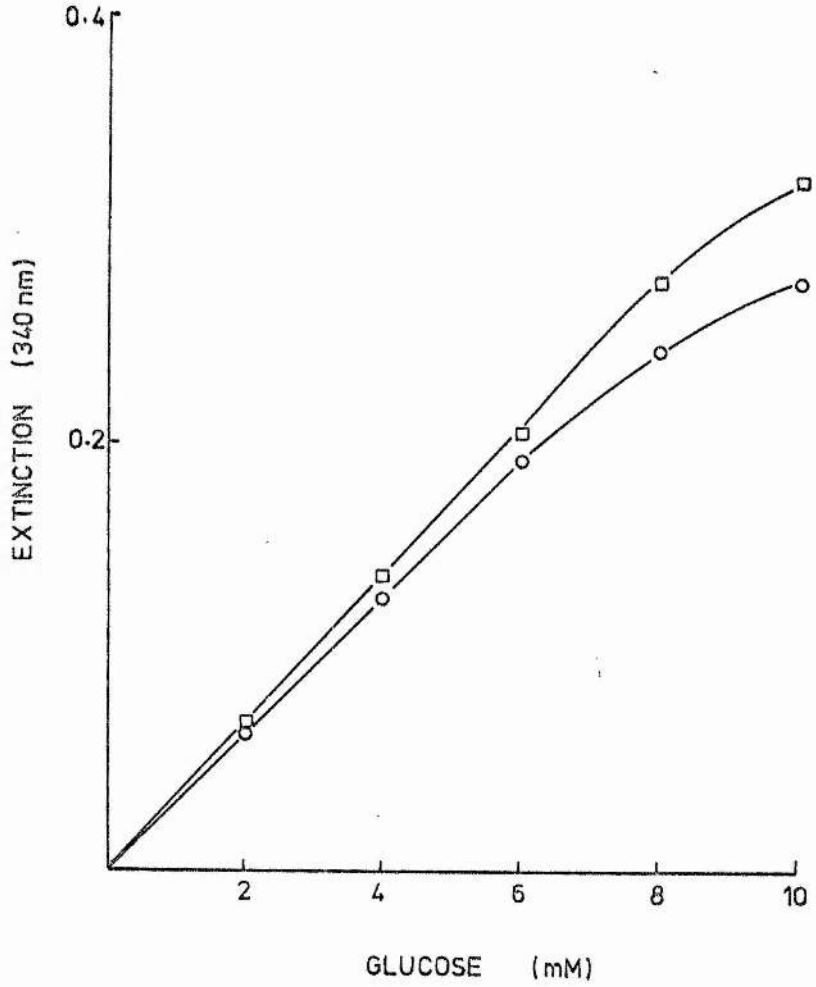

0.4
0.2
EXTINCTION (340 nm)
2
4
6
8
10
GLUCOSE (mM)

Fig 43(a) and 43(b) . Recorder traces obtained from the determination of 2-10 mM-glucose at sampling rates of 60 per h using nylon-tube immobilised derivatives of glucose oxidase , catalase and aldehyde dehydrogenase in series. In each case the first two derivatives were prepared by glutaraldehyde-activation and HMDA-substitution. The third derivative was prepared by diethyladipimidate-activation and (a) Adipic acid dihydrazide-substitution ; (b) HMDA-substitution.

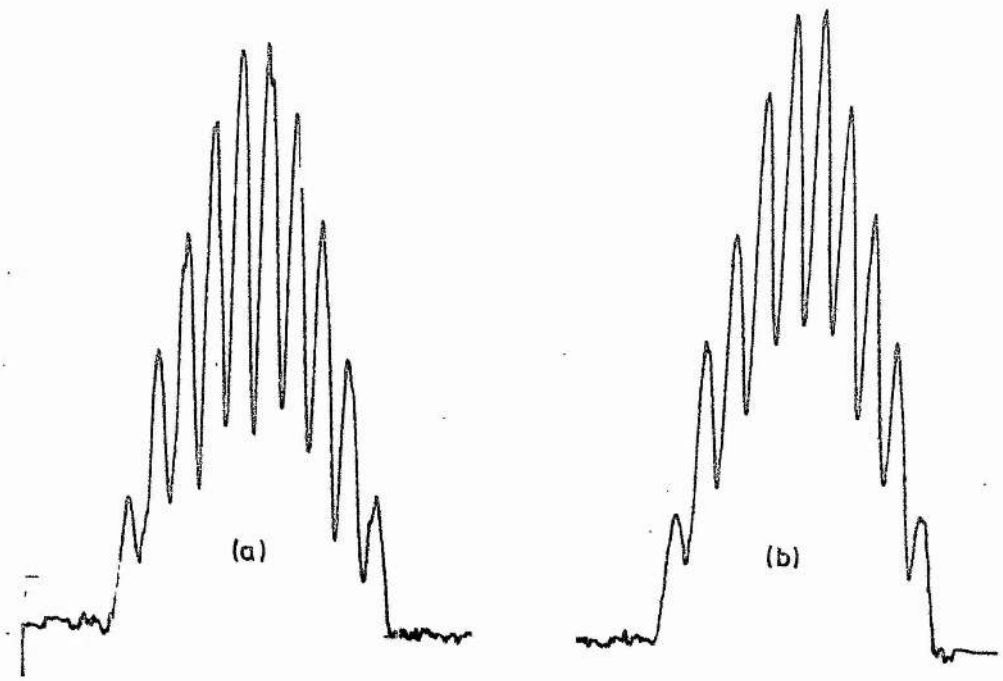

(a)
(b)

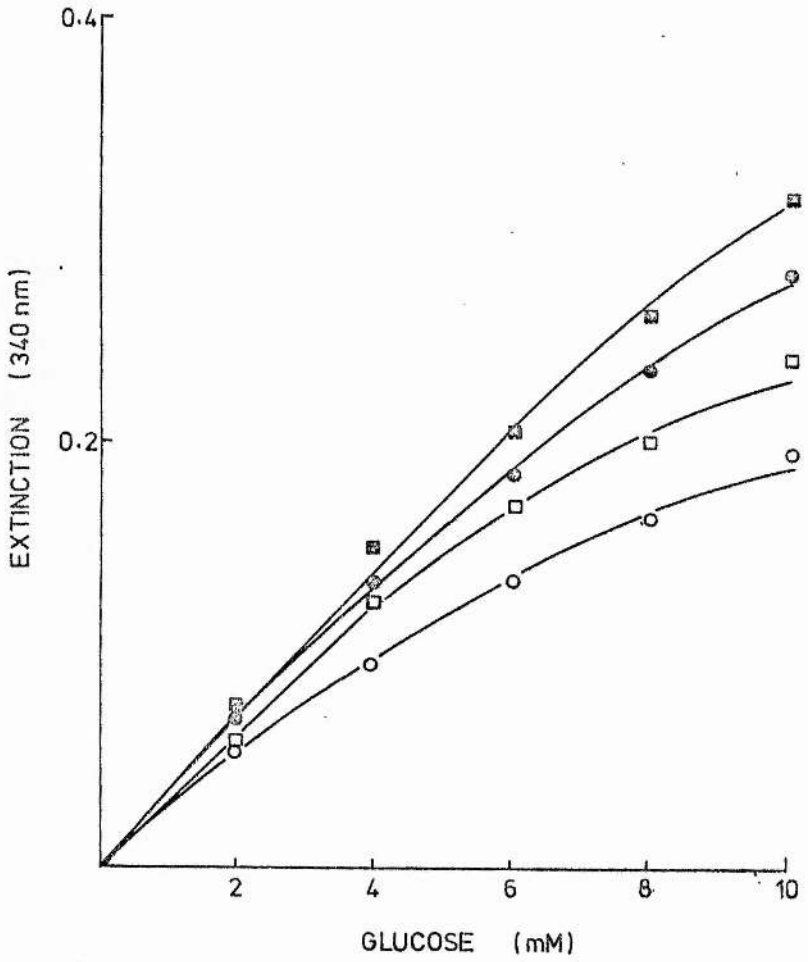
Fig 44 . Effect of continuous serum assays upon the combined activity of nylon-tube immobilised derivatives of glucose oxidase, catalase and aldehyde dehydrogenase in series.

▨ Combined activity immediately after preparation

◍ After 810 serum assays

□ After 1 440 serum assays

○ After 2 200 serum assays


0.4
0.2
EXTINCTION (340 nm)
2
4
6
8
10
GLUCOSE (mM)

bore aldehyde dehydrogenase tube. In each case 2–10 mM-glucose samples were analysed at a sampling rate of 60 per h and the appropriate standard curves compiled. The results of these experiments and the recorder traces are shown in Figs 42 and 43a–b and indicate that glucose concentrations as low as 2 mM may be analysed at sampling rates of 60 per h.

However, the peak geometries obtained with a dihydrazide-substituted aldehyde dehydrogenase tube exhibited less carry over than those obtained using a corresponding diamine-substituted derivative. This difference in the adsorptive character of the two derivatives can be rationalised along the lines used in section 4.1.9 to explain their different behaviour to protein adsorption. This observation suggests that although the dihydrazide-substituted nylon-tubes display less activity than diamine-substituted derivatives, the operational performance may be superior in autoanalysis.

The operational stability of the system was investigated in the following manner. 2 m lengths of the three derivatives were inserted into the flow system described in Fig 13 and a standard curve for glucose compiled by the analysis of 2–10 mM-glucose samples as described above. Serum samples were then assayed at a rate of 60 per h for a period of 36 h. At intervals, further standard curves were compiled in order to monitor the combined activity of the three derivatives. The results of these experiments are shown in Fig 44 and indicate that although the combined activity of the three derivatives decreased by 33% over 2 200 serum assays, sufficient activity remained to ensure the sensitivity of further serum glucose determinations. Therefore this assay protocol for the determination of glucose is capable of performing at least 2 200 serum assays.

5.4. DETERMINATION OF GLUCOSE USING NYLON-TUBE CO-IMMOBILISED GLUCOSE OXIDASE AND CATALASE IN CONJUNCTION WITH A FLOW-THROUGH OXYGEN ELECTRODE

For every mole of glucose converted to δ-gluconolactone in the presence of glucose oxidase, one mole of oxygen is utilised and one mole of H_2O_2 produced. Previous assay procedures for the determination of glucose have involved the monitoring of H_2O_2. However, Campbell et al have described a system for the autoanalytical determination of glucose where the oxygen utilised by a nylon-tube immobilised glucose oxidase derivative is measured using a flow-through oxygen electrode. This assay system can lead to errors in glucose estimation owing to the presence of catalase, endogenous to erythrocyte cells (22). The partial conversion of H_2O_2 to oxygen realised in this manner, results in an oxygen : glucose stoichiometry of less than one. However, the use of glucose oxidase in conjunction with excess catalase has been shown to completely remove this source of error by reducing the oxygen : glucose stoichiometry from 1 : 1 to 0.5:10 (22). It has been previously shown in section 5.1.2 that the catalitic activity associated with a nylon-tube co-immobilised glucose oxidase and catalase derivative is in excess of the immobilised glucose oxidase activity. This suggests that the derivative may be used for the determination of glucose using a flow-through oxygen electrode. This was investigated in the following manner.

A 3 m length of HMDA-substituted/ 1 mm-bore nylon-tube co-immobilised glucose oxidase and catalase derivative was prepared as described previously. The enzyme coupling solution comprised 2 mg ml^{-1} catalase and 1 mg ml^{-1} glucose oxidase in 0.2 M-phosphate, pH 7.8. A glucose standard curve was compiled by the insertion of the derivative into the flow system described in Fig 15, and the analysis of 2-10mM-glucose samples at a sampling rate of 60 per h as described in section 3.6.11.

Fig 45 . Standard curve for the estimation of glucose using a GOD/CAT derivative in conjunction with a flow-through oxygen electrode.

Fig 46 . Effect of 10 000 aqueous glucose assays upon the activity of a GOD/CAT derivative when used in conjunction with a flow-through oxygen electrode.

- O Combined activity immediately after preparation
- □ After 10 000 assays

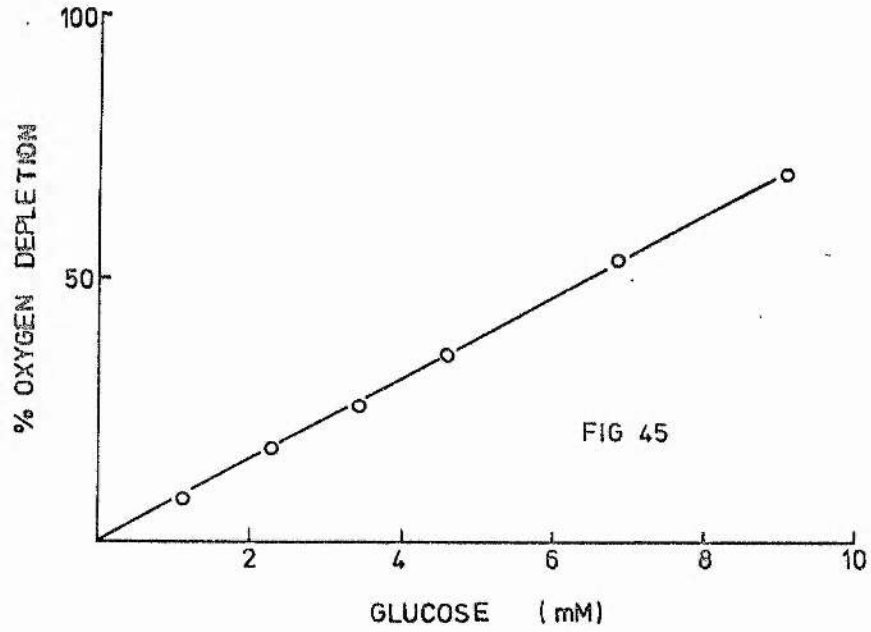

100
50
% OXYGEN DEPLETION
2
4
6
8
10
GLUCOSE (mM)

FIG 45

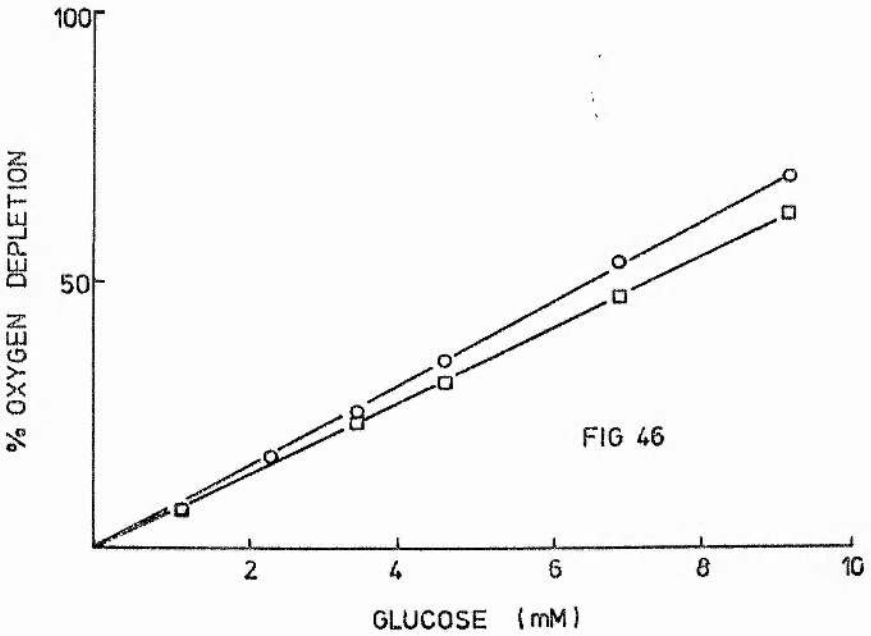
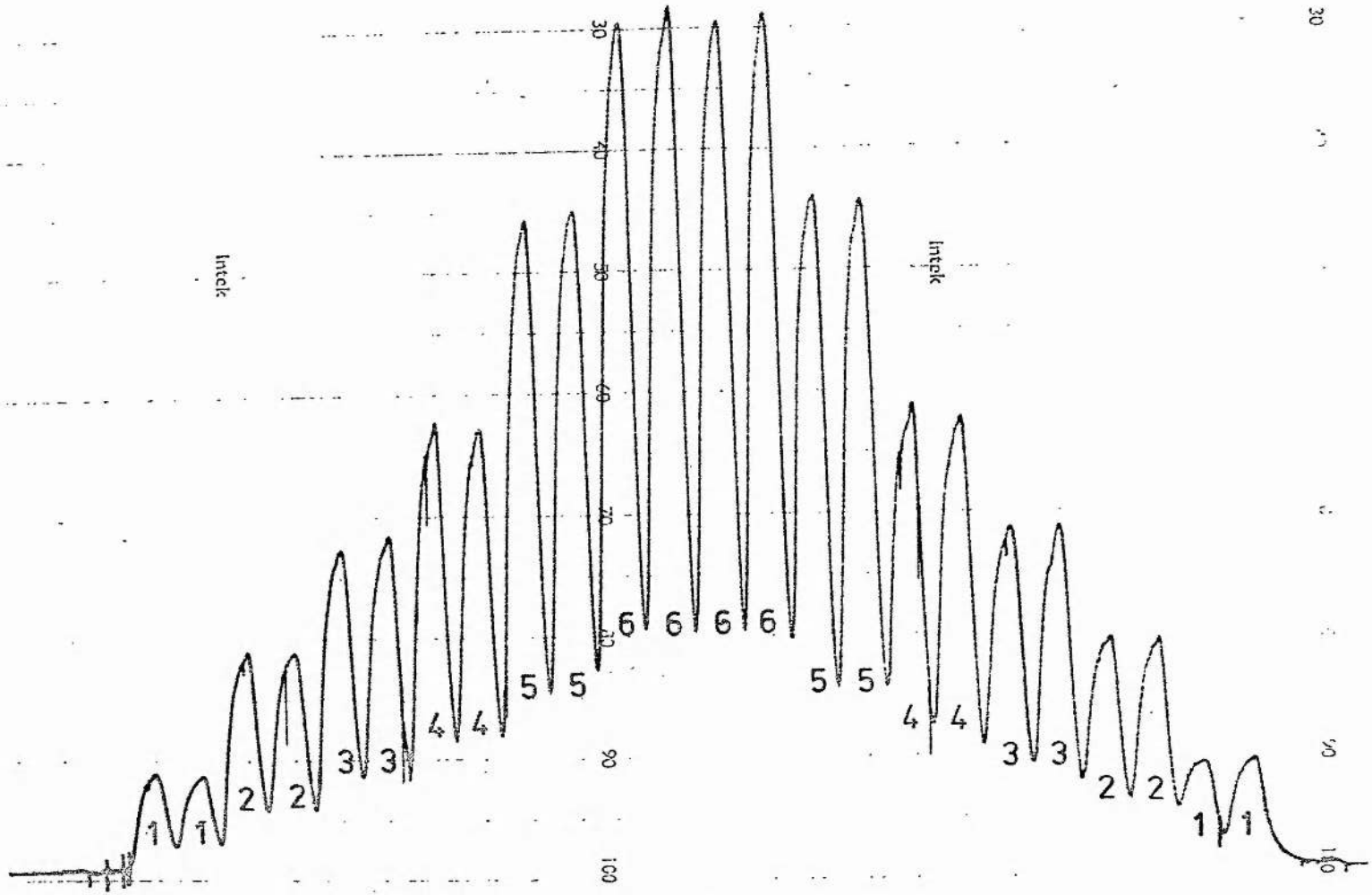

100
50
% OXYGEN DEPLETION
2
4
6
8
10
GLUCOSE (mM)

FIG 46

Fig 47 . Recorder trace obtained from the determination of 2-10 mM-glucose at a sampling rate of 60 per h using a COD/CAT derivative in conjunction with a flow-through oxygen electrode.


Intak
Intak
1 1 2 2 3 3 4 4 5 5 6 6 6 6 5 5 4 4 3 3 2 2 1 1
40
30
20
10
0

The results of this experiment are shown in Fig 45 and indicate that the assay system exhibits sufficient sensitivity for the accurate determination of glucose concentrations as low as 1 mM.

A typical recorder trace obtained by the analysis of duplicate samples of 2-10 mM-glucose at a sampling rate of 60 per h is shown in Fig 47. This result indicates the absence of any marked analytical errors due to cross-contamination of samples in the flow stream of the analyser when glucose is assayed at this sampling rate.

The stability of the GOD/CAT tube under operational conditions was examined in the following manner. A standard curve for glucose was first compiled in the manner described above. Glucose samples were then assayed at sampling rates of 60 per h for a period of one week during which time over 10 000 continuous assays were performed. A further standard curve was then compiled in the manner described above. The results of this experiment are shown in Fig 46 and indicate that only a 10% loss in activity occurred over this period. This indicates that a GOD/CAT derivative may be used for over 10 000 glucose assays by this method.

Thus, determination of glucose using a nylon-tube co-immobilised derivative of glucose oxidase and catalase in conjunction with a flow-through oxygen electrode meets the standards of sensitivity, stability, and speed of operation that are required for clinical analytical systems.

SUMMARY OF CHAPTER 5

Four different assay protocols for the determination of glucose have been described. All involved the primary enzymic reaction of glucose oxidase. The first two systems incorporated separately immobilised derivatives and coimmobilised derivatives of glucose

oxidase and catalase respectively. These systems were found to exhibit the necessary sensitivity, stability and speed of operation required of clinical autoanalytical systems. However, DMS-alkylation of nylon-tubes produced derivatives that exhibited less activity than the corresponding TOTFB-alkylated derivatives and were not capable of analysing glucose samples at the required rate. For these reasons they were regarded as unsuitable for incorporation into analytical systems.

The third method for the estimation of glucose linked the peroxidatic activity of catalase to a system that could be monitored spectrophotometrically at 340 nm. This was achieved by means of the addition to the system of a length of nylon-tube immobilised aldehyde dehydrogenase. The most active derivative of the latter enzyme involved adipimidate activation of a HMDA-substituted 2 mm bore nylon-tube. Glutaraldehyde activation in this instance produced an enzyme derivative displaying 87% less activity. The assay system was capable of operating at sampling rates of 60 per h. A 33% decrease in the combined activity of nylon-tube immobilised derivatives of glucose oxidase, catalase and aldehyde dehydrogenase occurred over 2 200 continuous serum assays although adequate combined activity remained to ensure the sensitivity of further assays.

The final assay protocol for the determination of glucose involved a nylon-tube co-immobilised glucose oxidase and catalase derivative in which the catalase moiety was used in a catalatic manner. This so-called "Reagentless" assay system depended upon monitoring depletion of dissolved oxygen by a flow-through oxygen electrode. It was observed to display the necessary sensitivity, stability and speed of operation that is required for clinical autoanalytical systems.

CHAPTER 6

GENERAL DISCUSSION

A Preparation and Properties of Nylon-Tube Immobilised Enzymes

The data presented in this thesis has consistently demonstrated that the TOTFB-alkylation of nylon-tubes produces more active immobilised derivatives than those obtained by DMS-alkylation. It seems likely that this is due to the greater yield of secondary imidates on the nylon produced by the former reagent. Whereas DMS only reacts with nylon at 100°C, the alkylation reaction of TOTFB proceeds at room temperature. The markedly different reaction conditions will affect secondary imidate formation, as the latter have been reported to be most sensitive to hydrolysis at elevated temperatures (49). As DMS alkylation of nylon is performed at 100°C, the presence of any water will adversely affect the secondary imidates formed. Thus use of TOTFB at room temperature should minimise any product hydrolysis. It is also possible that the DMS may adversely affect the reaction due to a solvent effect upon the nylon. It has been observed previously that DMS-alkylation of nylon-tube can cause blockages in the tubes due to precipitation of nylon during the reaction process (48). Thus a situation might arise whereby secondary imidate formation is hindered by the solubility of the nylon in DMS.

Although TOTFB-alkylation has been shown to be a far superior method for nylon activation than DMS-alkylation, problems still arise over its use. Not only is TOTFB difficult to obtain commercially, but also it has limited storage stability. The use of alternative reagents may overcome these problems. For example, p-toluene sulphonyl chloride can convert secondary amides to imidoyl chlorides (Fig 48). This suggests that it may be of use in the generation of imidoyl chloride functions on the surface of a nylon-tube. These could then be reacted with diamines or acid dihydrazides, in a similar manner to imidates, to form amidines and amidrazones respectively. Activation

in this manner may therefore overcome problems associated with TOTFB activation of nylon-tube.

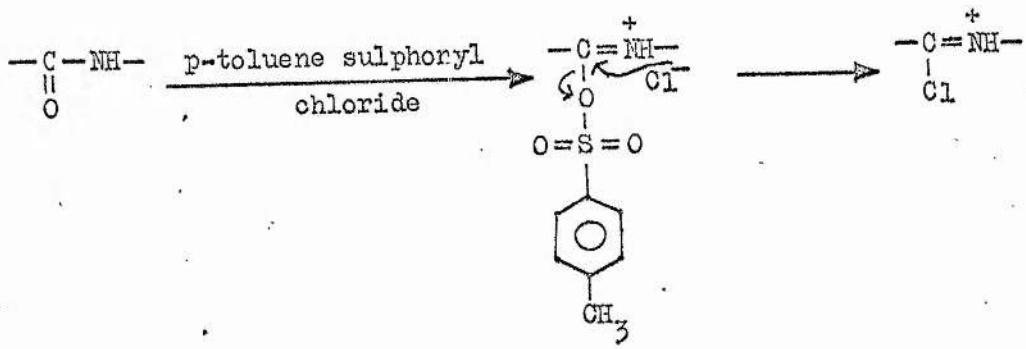


Fig 48. Activation of Secondary Amides using p-Toluene Sulphonyl Chloride

Although catalase was found to be inactive when bound directly onto the O-alkylated nylon-tube, activity was retained by substitution of the nylon with such compounds as diamines, acid dihydrazides, and inert protein. Although in this thesis the types of spacer compounds employed in the preparation of immobilised enzymes have been relatively few in number, a great variety of different compounds can be employed as spacers in a similar manner. For example, use of 4,4'-methylenedianiline as a spacer would introduce aryl amino groups into the support ; use of glycine or glutamic acid would result in carboxyl group introduction ; and use of cysteamine would introduce thiol groups into the support. This flexibility of support chemistry may be of benefit when considering the insolubilisation of different enzymes. One set of immobilisation conditions suitable for one particular enzyme may not be applicable to another, and therefore a variety of support chemistries may be needed.

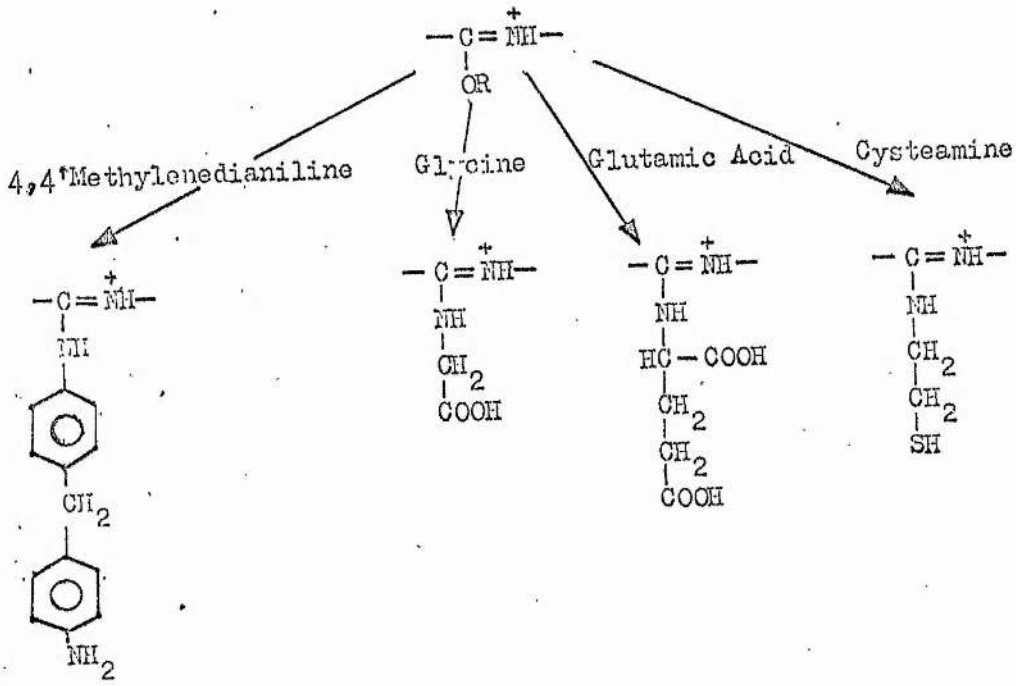


Fig 49. Alternative Substitutions of Nylon-tube.

Of the spacers examined in this thesis, diamines consistently bound more protein than acid dihydrazides. However, the smaller amounts of bound protein associated with the hydrazide-substituted nylon-tubes may only be due to the relative insolubility of the latter compounds in the organic solvents used in the substitution reaction the limited quantity of acid dihydrazides proffered to the activated nylon-tube may affect the extent of the substitution reaction. Another effect contributing to the lower bound protein content of these derivatives will be the absence of any adsorbed protein. Dihydrazide-substituted nylon-tube immobilised enzymes have been shown to be far 'cleaner' derivatives than the corresponding diamine-substituted derivatives owing to the lack of the positive charge associated with the amidine link which causes the adsorption of both protein and coenzymes onto the support in the case of amine-substituted nylon tubes. Therefore, in terms of applications, hydrazide-substituted derivatives may be superior to the corresponding diamine-substituted

tubes if the solubility of dihydrazides can be increased by choice of an alternative organic solvent. For example, in the field of autoanalysis, the operational rate of any system is adversely affected by non-specific adsorption. Use of hydrazide-substituted enzyme tubes may allow the operating rates to be increased by decreasing sample cross-contamination. Hydrazide-substituted supports may also be useful in the field of solid phase immunology. When considering the immobilisation of antibodies to a support for use in immunoassays, the immobilised derivative must not cause non-specific adsorption of proteins otherwise serious errors may arise. For these reasons diamine-substituted supports are clearly unsuitable whereas dihydrazide-substituted supports may be of use.

The third stage of the immobilisation of enzymes to nylon-tube is the activation of the substituted nylon-tube. The majority of immobilised derivatives examined in this thesis were prepared by either glutaraldehyde- or diethyladipimidate-activation of the support. It has been shown that use of the latter reagent in place of glutaraldehyde generally results in an increase in immobilised enzyme activity. In the case of catalase, this increase was only 6%, whereas a seven-fold increase was obtained for immobilised aldehyde dehydrogenase. Thus the use of glutaraldehyde in the immobilisation of aldehyde dehydrogenase adversely affects the activity of the resulting derivative. This may be caused by either its non-specificity or the chemical bonds formed. In the former case, non-specificity of the reagent may unneccessarily involve amino-acids that are essential for the catalytic activity of the enzyme. In the latter case the removal of the positive charge of a protonated amine group involved in reaction with glutaraldehyde may cause the deactivation of the enzyme. On the other hand, diethyladipimidate will preserve the

positive charge when reacting with such a group and therefore may preserve the enzymic activity by maintaining the charge balance of the enzyme molecule. (Fig 50).

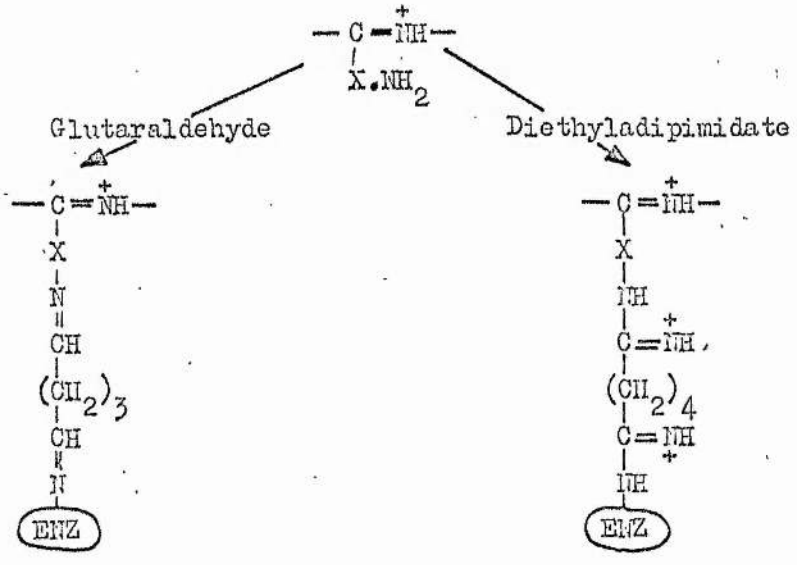


Fig 50. The Immobilisation of Enzymes using Glutaraldehyde and Diethyladipimidate.

The marked differences exhibited by catalase and aldehyde dehydrogenase in their susceptibility to glutaraldehyde highlights the need for diverse chemistries for the immobilisation of different enzymes.

The reactivation of the substituted support for the attachment of enzymes is not limited to the two reagents discussed so far. There exists a plethora of ways in which such reactivation may be achieved. For example, diethyladipimidate is just one of a large number of bis-imidates. The use of longer chain bis-imidates will result in increases in the displacement distance of enzymes from the support, and this may cause further increases in the retention of immobilised activity. Alternatively, such compounds as dibromoacetone, diisothiocyanate or trichloro-s-triazine may be suitable for use in the attachment of enzymes to diamino- and dihydrazide-substituted supports. As

previously discussed, substitution of nylon is not limited to these two types of compounds, and therefore alternative activation procedures may be necessary. For example, a glutamic acid- or glycine-substituted support could be reactivated through the carboxyl group by carbodiimides. Alternatively such substitutions as acid dihydrazides or aromatic compounds containing para-amino functions could be activated for enzyme attachment by the generation of acid azides or diazonium salts respectively with ice-cold nitrous acid, in so doing avoiding the use of an extra bifunctional reagent. These examples serve to indicate the diversity of methods available for the activation of substituted supports.

Any discussion of immobilisation techniques should also include the possible presence of diffusional effects. Although in solution, diffusion occurs so rapidly that only a few reactions are fast enough to be restricted by it, the reverse is thought to be true for many heterogeneous catalysts where only very slow enzymic reactions may escape diffusional restrictions (64). Catalase is an enzyme with a high turnover number and its reaction rate may be considered likely to be limited by diffusion in an immobilised form. It has been shown that immobilisation of larger quantities of catalase upon nylon-tubes does not increase the overall tube activity, but appears to increase the stability of the enzyme to inactivation by H_2O_2. The former effect has previously been discussed with regard to overcrowding of enzyme molecules and the finite number of binding sites located on the surface of the nylon-tube. However, both effects can also be explained by the presence of diffusional restrictions. Several immobilised enzymes have been reported to be under diffusional control. For example, the reaction catalysed by nylon-tube immobilised asparaginase has been shown to be largely controlled by diffusion at low flow rates

and low substrate concentrations (65). Two different types of diffusional restrictions have been reported for immobilised enzymes (66). Film diffusion occurs when substrate molecule in the bulk solution have to diffuse through the quasi Nernst diffusion layer to reach the catalytic surface. Pore diffusion occurs when the substrate at the surface of the support has to diffuse into the support in order to approach the enzyme. In the case of nylon-tube immobilised enzymes prepared by O-alkylation of the nylon, the extent of this latter type of diffusion is thought to be minimal owing to the immobilisation chemistry employed. This is thought to immobilise the majority of enzyme upon the surface of the nylon. However, film diffusion has been shown to be present in open tubular heterogeneous reactors at flow rates similar to those employed in this work for the determination of the stability of nylon-tube immobilised catalase derivatives (34,35).

The presence of diffusional restrictions upon the rate of conversion of H_2O_2 by immobilised catalase could explain both the absence of increased activity and the observed stabilisation of the derivative on increasing the quantity of catalase bound to the support. In the presence of diffusional control, increasing the levels of bound enzyme above a critical value will not be reflected by an increase in the immobilised enzyme activity. However, if only a percentage of the active enzyme bound to the support was responsible for that activity, a situation might arise where any enzyme molecules rendered inactive could be immediately replaced by enzymes hitherto uninvolved in the overall reaction rate. This behaviour might also explain the unusual inactivation pattern exhibited by immobilised catalase derivatives. In the situation envisaged above, catalase

tubes could be expected to show stability until all of the active immobilised enzyme is involved in the enzymic reaction. At this point, the immobilised activity will decrease rapidly owing to as when all the active immobilised enzyme is participating in the reaction, there will be no possibility of replacement of inactive enzyme. Therefore this hypothesis might explain the experimentally obtained time-course of deactivation of nylon-tube immobilised catalase.

The instability of immobilised catalase in the presence of H_2O_2 is thought to be due to the formation of an inactive H_2O_2 - catalase complex. This may be a form of either (or both) of the so-called Compounds 2 or 3 that are known to be formed by the soluble enzyme in the presence of excess H_2O_2 (23). Several other similarities in the behaviour of the soluble and immobilised enzymes have been observed : 1) Each displays competitive peroxidatic and catalatic activities 2) In each case the maximum rate of peroxidatic activity is achieved in the presence of 2.5 M-methanol ; 3) Both lose activity in the presence of H_2O_2 and are more stable in its absence 4) Both exhibit 'pH optima' around neutrality (although the pH dependence of the immobilised enzyme is reduced). The ability of the immobilised enzyme to mimic the soluble enzyme in this manner can imply the ability of the immobilised catalase to form complexes similar to the compounds 2 and 3 of the soluble enzyme. However, it must be emphasised that the formation of these compounds by the immobilised catalase has not been proved experimentally.

B Autoanalysis using Nylon-Tube Immobilised Enzymes

The autoanalysis of glucose can be performed by enzymic or chemical methods. As previously discussed, the use of enzymes in metabolite analysis achieves a higher degree of assay specificity than that obtained using chemical reactions. Several assays involving soluble enzymes have been proposed for the analysis of glucose. The so-called Trinder method involves the linked reaction of glucose oxidase and peroxidase (7), while another method involves the linked reaction of hexokinase and glucose-6-phosphate dehydrogenase (8). The former system has been criticised due to the ability of peroxidase to accept a number of compounds as its second substrate, thus increasing the possibility of assay interference from substances such as ascorbic acid that may be present in blood. At the present time the glucose oxidase/peroxidase method cannot be converted into an assay system involving the corresponding nylon-tube immobilised enzymes owing to the gross adsorption of the second substrate for peroxidase onto the surface of the nylon-tube. This not only decreases the sensitivity of the assay but reduces its operational rate to about 20 samples per h, a rate that is completely unacceptable in an automated assay for glucose (68,69).

The autoanalysis of glucose has been suggested using nylon-tube immobilised derivatives of hexokinase and glucose-6-phosphate dehydrogenase (46). Two disadvantages are associated with this system : 1) use of the coenzymes NADP and ATP increase the operational costs of the system and 2) the stability of the enzyme derivatives is poor, only 3,500 serum assays being performed per derivative.

Four systems have been described in this thesis for the automated determination of glucose, and each has been shown to satisfy some of the standards required for clinical analytical systems.

The first system utilised a nylon-tube co-immobilised derivative of glucose oxidase and catalase. While the co-immobilisation of these enzymes ensures the presence of conditions favoring the peroxidatic activity of catalase, it is also possible that a second enhancement of the combined activity may occur owing to the close proximity of each enzyme. Mosbach and Mattiasson have reported such an enhancement of the combined activity of hexokinase and glucose-6-phosphate dehydrogenase co-immobilised to the same support (67).

However, replacement of individual immobilised enzymes is impossible in this system, and the situation may arise where one immobilised enzyme retains its activity after the other is inactivated. In this case the enzyme derivatives could not be used in the most economical fashion. In addition, this system could not be used for monitoring other oxidase reactions such as urate oxidase, where wide discrepancies exist in the optimal pH for storage and activity of catalase and urate oxidase.

The second assay system, by incorporating separately immobilised derivatives of glucose oxidase and catalase permits individual replacement of derivatives, thus increasing the cost-effectiveness of the system. The separation of the two enzymes onto different nylon-tubes does lead to a 20% decrease in combined activity. However, this is recovered by the use of 2 mm bore nylon-tube immobilised derivatives in place of 1 mm bore nylon-tube enzymes.

This assay system allows the comparison of enzyme requirement per assay for soluble and immobilised enzyme analyses. The Trinder method uses soluble glucose oxidase as the primary enzyme in glucose analysis. Whereas this method uses 16.6 IU of glucose oxidase per assay, it has been shown that a GOD/CAT derivative displaying 5.5 IU

of glucose oxidase activity is capable of performing at least 10,000 serum assays. This emphasises the great conservation of enzyme that is achieved using nylon-tube immobilised enzymes in clinical analysis.

However, two disadvantages are associated with the Hantzsch reaction for the measurement of the peroxidatic activity of catalase: 1) the slow rate of the chemical reaction requires the inclusion into the flow system of a 30 turn delay coil in order to allow colour to develop and 2) since it is a chemical reaction, the Hantzsch reaction may be susceptible to interference from substances present in blood. The former point renders both systems less suitable for the latest designs of autoanalyser which are being designed to attain high sampling rates and low sample residence time. The chemical nature of the Hantzsch reaction makes the whole assay system more susceptible to interference and lowers the specificity gained by the use of enzymes.

The third system, incorporates separately immobilised derivatives of glucose oxidase, catalase and aldehyde dehydrogenase placed in series. This assay displays three advantages over the previous systems : 1) glucose may be determined spectrophotometrically at 340 nm, 2) The involvement of immobilised aldehyde dehydrogenase eliminates the requirement for a 30 turn delay coil in the system, making it more suited to the new range of autoanalysers : 3) The aldehyde dehydrogenase reaction is probably more specific than the chemical Hantzsch reaction.

However, this system possesses only 30% of the sensitivity exhibited by the Hantzsch reaction assays, and displays comparatively poor operational stability. Whereas only 22% activity loss was observed over 10,000 serum assays by the co-immobilised GOD/CAT tube,

33% of the activity of the 340 nm linked assay was lost over 2,200 serum assays. However, only 12 IU of aldehyde dehydrogenase were used to perform these assays. In the soluble form it could be expected that at least 2 IU of the enzyme would be required for every assay.

Several possibilities exist for the improvement of a glucose assay using nylon-tube immobilised derivatives of glucose oxidase, catalase and aldehyde dehydrogenase. Although the operational conditions favoring the stability and combined activity of immobilised glucose oxidase and catalase derivatives have been studied in some detail, no such work has been performed with aldehyde dehydrogenase. For example, greater combined activity may be achieved by using longer lengths of the latter derivative. The requirements of soluble aldehyde dehydrogenase are fairly complex as it is an enzyme that requires thiols and is activated by potassium ions. This further suggests that optimisation of the operational conditions of the immobilised enzyme may produce improvements in its stability and activity. A further possibility existing for the improvement of the 340 nm linked glucose assay is the purification of the aldehyde dehydrogenase from a different source. As in the case of fungal catalase, aldehyde dehydrogenase isolated from a source such as the saccharolytic Clostridia may have improved stability.

The last system described in this thesis is the so-called 'Reagentless' assay of glucose. It is perhaps the most attractive method of the four discussed owing to its extreme simplicity. The absence of any requirement for colour reagents or coenzymes ensures the economic superiority of the system. However, flow-through oxygen electrodes are not as yet commercially available, and this need for new equipment may mar the attractiveness of the system at the present time. It is to be hoped that the attractiveness of the system may well

stimulate the commercial production of such apparatus.

The systems presented in this thesis for the determination of glucose all involve the monitoring of glucose oxidase activity. This is achieved by either analysis for its product, H_2O_2 or depletion of one of its substrates, dissolved oxygen. Therefore in principle every method described for the monitoring of the latter reaction can be applied to any other metabolite analysis that involves an oxidase as the primary enzymic reaction. This group of analyses involves several important metabolite assays such as Uric Acid (by urate oxidase), Ethanol (by alcohol oxidase) and Cholesterol (by cholesterol oxidase).

This implies that if the primary enzyme can be immobilised, the assay procedures involving immobilised derivatives described in this thesis can be used to monitor the appropriate enzyme. The requirements of any two oxidases are often slightly different and thus slight modifications may be required. For example, urate oxidase displays a pH optimum around pH 8.5. Unless the pH of the reagent stream is changed after the urate oxidase reaction, immobilised fungal would be required as the beef-liver enzyme would not exhibit enough stability at that pH. In the case of alcohol oxidase, difficulties might be encountered in the ability of catalase to utilise ethanol as a substrate. However, this may afford a means of estimating ethanol without recourse to an enzyme that is not commercially available at the present time. The determination of cholesterol should present no difficulties with catalase stability or specificity but will require the presence of a further immobilised enzyme, cholesterol esterase. This system would then be capable of estimating total cholesterol levels in the blood.

BIBLIOGRAPHY

19 Traber, A.D. and Kittrell, J.R. (1974), Biotechnol. Bioeng., [illegible]

20 Altomare, R.E., Kohler, J., Greenfield, P.F. and Kittrell, J.R. (1974), Biotechnol. Bioeng., 16, 1659.

21 Jaworek, D. (1973), Engineering Foundation Conference, Henniker USA.

22 Dritschilo, W. and Weibel, M.K. (1974), Biochem. Med., 9, 32.

23 Deisseroth, A. and Dounce, A.L. (1970), Physiol. Rev., 50, 319.

24 Kageyama, N. (1971), Clin. Chim. Acta, 31, 421.

25 Nash, T. (1953), Biochem. J., 55, 416.

26 Datta, S. and Grzybowski, A. (1961) , in Biochemist's Handbook. p 19.

27 Vogel, A.I. (1953), in Macro and Semimicro Qualitative Inorganic Analysis, 4th Edition, p 414, Longmans, Green and Co., London.

28 Bergmeyer, H.U. (1963), Methods of Enzymatic Analysis, p 290 1st Edition, Academic Press, London and New York.

29 Meerwein, H. (1966), Org. Syn., 46, 113.

30 Pinner, A. (1892), Die Impoather und ihre Derivative, Oppenheim, Berlin.

31 Bergmeyer, H.U. (1963), Methods of Enzymatic Analysis, p 886, 1st Edition, Academic Press, Londod and New York.

32 Bergmeyer, H.U. (1963), Methods of Enzymatic Analysis, p 293, 1st Edition, Academic Press, London and New York.

33 Ford, J.R., Lambert, A.H., Cohen, W. and Chambers, R.P. (1972), Biotechnol. Bioeng., Symp. No. 3, p 267.

34 Koyayashi, T. and Laidler,K.J. (1974), Biotechnol. Bioeng., 16, 99.

35 Horvath, C. and Solomon, B.A. (1972), Biotechnol. Bioeng., 14, 885.

1. Goldman, R., Goldstein, L. and Katchalski, E. (1971), in Biochemical Aspects of Reactions on Solid Supports, Edited by Stark, G., Academic Press, London.

2. Miyamoto, K., Fujii, T. and Miura, Y. (1971), J. Ferment. Technol., 49, 565.

3. Melrose, G.J.H. (1971), Rev. Pure and Appl. Chem. 21, 83.

4 Hornby, W.E., Inman, D.I. and Mcdonald, A. (1972), FEBS Lett., 23, 114.

5 Weetall, H.H. (1973), Food Product Development, 12, 22.

6 Weetall, H.H. (1971), Research and Development, 22, 18.

7 Trinder, P. (1969), Ann. Clin. Biochem., 6, 24.

8 Widdowson, G.M. and Penton, J.R. (1974), Clin. Chem. (Winston-Salem) 18, 299.

9 Martinek, R.G. (1969), J. Am. Med. Technol., 31, 530.

10 Filippusson, H., Hornby, W.E. and Mcdonald, A. (1972), FEBS Lett., 20, 291.

11 Inman, D.J. and Hornby, W.E. (1972), Biochem. J., 129, 255.

12 Weetall, H.H. (1970), Biochim. Biophys. Acta, 212, 1.

13 Silman, I.H. and Katchalski, E. (1966), A. Rev. Biochem., 35, 873.

14 Warburton, D., Dunnill, P. and Lilly, M.D. (1973), Biotechnol. Bioeng., 15, 13.

15 Hofstee, B.H.J. (1973), Biochem. Biophys. Res. Comm., 50, 751.

16 Balcom, J., Foulkes, P. and Olson, N.F. (1971), Process Biochem., 6, 42.

17 Traher, A.D. and Kittrell, J.R. (1974), Biotec. Bioeng., 16, 419.

18 Brown, E. and Joyeau, R. (1974), Macromol. Chem., 175, 1961.

ADDENDUM

The term K_{app} is defined as the apparent rate constant, where :-

$$K_{app} = \frac{k_t \cdot L}{K}$$

Where k_t = Tube specific activity

L = Length of tube

K = Michaelis constant

36 Campbell, J., (1974), Ph.D Thesis, St. Andrews University.

37 Campbell, J., Hornby, W.E. and Morris, D.M. (1975), Biochim. Biophys. Acta, 384, 307.

38 Onyzeli, F. (1974), MSc Thesis, St. Andrews University.

39 Hornby, W.E., Lilly, M.D. and Crook, E.M. (1966), Biochem. J. 98, 420.

40 Hornby, W.E. and Filippusson, H. (1970), Biochim. Biophys. Acta, 220, 343.

41 Hornby, W.E., Filippusson, H. and Mcdonald, A. (1970), FEBS Lett., 9, 8.

42 Sundaram, P.V. and Hornby, W.E. (1970), FEBS Lett., 10, 325.

43 Inman, D.I. and Hornby, W.E. (1974), Biochem. J., 137, 25.

44 Hornby, W.E. and Morris, D.L. (1973), U.K. Patent No. 37230/73.

45 Richards, F.M. and Knowles, J.R. (1969), J. Mol. Biol., 37, 231.

46 Morris, D.L., Campbell, J. and Hornby, W.E. (1975), Biochem. J., 147, 593.

47 Hornby, W.E. and Morris, D.L. (1975), in Immobilised Enzymes, Antigens, Antibodies and Peptides, p 141, Edited by Weetall, H.H., Marcel Dekker Inc., New York.

48 Campbell, J. (1974), Ph.D Thesis, p 47, St. Andrews University.

49 Roger, R. and Neilson, D.G. (1961), Chem. Rev., 61, 179.

50 Jost, R., Miron, T. and Wilchek, M. (1974), Biochim. Biophys. Acta, 362, 75.

51 Morris, D.L. and Hornby, W.E. , Personal communication to the author.

52 Nicholls, P. (1962), Biochim. Biophys. Acta, 60, 217.

53 Scott, D. and Hammer, F. (1960), Enzymol., 22, 229.

54 The Enzymes. Edited by Boyer, P.D., Lardy, H. and Myrback, K., Vol 8, p 261, Academic Press, London and New York.

55 Chance, B. (1947), Acta Chem. Scand. 1, 236.

56 Kremer, M. (1970), Biochim. Biophys. Acta, 198, 199.

57 Zidoni, E. and Kremer, M. (1974), Arch. Biochem. Biophys.,161, 658.

58 Chance, B., Greenfield, S. and Roughton, J.W. (1952), Arch. Biochem. Biophys., 37, 301.

59 Oshino, N., Oshino, R. and Chance, B. (1973), Biochem. J., 131, 555.

60 Keilin, D. and Hartree, E.F. (1945), Biochem. J., 39, 293.

61 Noy, G.A. (1975), Personal communication to the author.

62 Chance, B. (1962), J. Biol. Chem., 194, 471.

63 Eastham, R.D. (1967), in Biochemical Values in Clinical Medicine, 3rd Edition, J. Wright and Sons Ltd., Bristol.

64 Laidler, K.J. and Bunting, P.S. (1973), in The Chemical Kinetics of Enzyme Action, p 382, 2nd Edition, Clarendon Press, Oxford.

65 Bunting, P.S. and Laidler, K.J., (1974), Biotechnol. Bioeng., 16, 119.

66 Rovito, B.J. and Kittrell, J.R. (1973), Biotechnol. Bioeng., 15, 143.

67 Mosbach, K. and Mattiasson, B. (1970), Acta Chem. Scand., 24, 2093.

68 Inman, D.I. (1973)., Ph.D Thesis, St. Andrews University.

69 Ashworth, S.D. (1974), Unpublished results.

70 Nilsson, H., Mosbach, R. and Mosbach, K. (1972), Biochim. Biophys. Acta, 268, 253.

71 Levin, Y., Pecht, M., Goldstein, L. and Katchalski, E. (1964), Biochem., 3, 1905.

72 Guilbault, G. and Lubrano, G.J. (1973),Anal. Chim. Acta, 64, 439.

73 Guilbault, G. and Lubrano, G.J. (1972), Anal. Chim. Acta, 60, 254.

74 Hicks, G.P. and Updike, S.J. (1966), Anal. Chem., 38, 726.

75 Enzyme Engineering Vol. 2, Edited by Pye, E.K. and Wingard, L.B., Plenum Press (1974), London and New York.

With the exception of 5α-androstane-3β,17β-diol, which stimulated only a weak proliferative response, the remaining metabolites elicited responses comparable to that of testosterone.

Using the same procedure, testosterone-induced DNA synthesis was examined as a potential model for evaluating the antiproliferative action of antiandrogens, oestrogens and hormone-cytotoxic agents. The potent antiandrogen, cyproterone acetate, exhibited a dose-dependent inhibitory effect on the testosterone-stimulated response. In chemically-defined organ culture, the hormone-cytotoxic agent, Estracyt^{R} (estramustine phosphate disodium and estramustine phosphate) and its derivative, estramustine, were consistently less effective than their carrier-hormone, oestradiol-17β, in suppressing the proliferative response to testosterone. However, in serum-supplemented medium, these compounds were all equally effective.

A method for the isolation of viable prostatic epithelial cells from rat ventral prostate was also established using a discontinuous Percoll^{R} density gradient. The epithelial origin of these cells was confirmed by the histochemical demonstration of acid phosphatase activity. A preliminary investigation of the growth potential of these cells in vitro was conducted using ^{125}I-UdR uptake and ^{3}H-TdR labelling coupled with autoradiography.

The potential value of normal rat ventral prostate in vitro as a model for assessing the effects of hormones and chemotherapeutic agents on prostatic growth is discussed, with particular reference to variations in culture media and methodology.